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FUNCTIONAL SIGNIFICANCE OF ADENOSINE IN CAROTID BODY CHEMOSENSORY ACTIVITY IN CONTROL AND CHRONICALLY HYPOXIC ANIMALS

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To my mum and dad

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- Conde S.V. and Monteiro E.C. **(2006),** Activation of nicotinic ACh receptors with $\alpha 4$ subunits induces adenosine release at the rat carotid body, Br. J. Pharmacol., 147, 783-789. (Chapter 2)

- Conde S.V. and Monteiro E.C. **(2004)**, Hypoxia induces adenosine release from the rat carotid body, J. Neurochem., 89 (5), 1148-1156. (Chapter 1)

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ABBREVIATIONS USED

5-HT- 5-hydoxytryptamine, serotonin

AADC - aromatic amino acid decarboxylase

AC – Adenylyl cyclase

ACh – Acetylcholine

AChE – Acetylcholinesterase

ADA – Adenosine deaminase

Ado – Adenosine

ADP- 5'-adenosine diphosphate

AK – Adenosine kinase

ANOVA – Analysis of variance

AMP – 5'-adenosine monophosphate

AOPCP - α,β-methylene ADP

ATP – 5'-adenosine triphosphate

CA – Catecholamine

cAMP – cyclic 5'-adenosine monophosphate

CB - Carotid body

CGS 21680 – 2-p-(2-Carboxyethyl)phenethyl-amino-5'-N-ethylcaboxamido-adenosine hydrochloride

ChAT – Choline acetyltransferase

CNT - Concentrative nucleotide transport

COPD – Chronic obstructive pulmonary disease

CSH – Chronic sustained hypoxia

CSN - Carotid sinus nerve

Cyt - Cytisine

DA – Dopamine

DBH – Dopamine–ß–hydroxylase

DH ßE – dihydro-ß-erythroidine

DMEM – Dulbecco's modified Eagle's medium

DMPP - dimethylphenylpiperazinium

DMSO – dimethylsulfoxide

DPCPX – 8-Cyclopentyl-1,3,-dipropylxanthine

E - Epinephrine

EHNA – erythro-9-(2-hydroxy-3-nonyl)adenine

Emax – maximal increase

ENT – Equilibrative nucleoside transport

ENT1 – Type 1 equilibrative nucleoside transport system

ENT2 – Type 2 equilibrative nucleoside transport system

GABA - Gamma aminobutyric acid

HE-NECA – 2-hexynyl-NECA

HPLC – High performance liquid chromatography

HVR – Hypoxic ventilatory response

L-DOPA – 3, 4,dihydroxy-L-phenylalanine

ME – met-encephalin

MRS 1754 – 8-4-{[(4-cyanophenyl)carbamoylmetyl]-oxy}phenyl)-1,3-di(n-propyl)xanthine

N-Apo – Propylnorapomorphine

NBTI - nitrobenzylthioinosine

NE – norepinephrine

NECA – 5'-(N-ethylcarboxamido)adenosine

Nic - Nicotine

NO – Nitric oxide

NT – Neurotransmitter

OSA – Obstructive sleep apnoea

PaO_2 – Partial arterial pressure of oxygen

$PaCO_2$ - Partial arterial pressure of carbon dioxide

PBS – Phosphate buffered saline

PG – Petrosal ganglion

PO_2 – Partial pressure of oxygen

RT-PCR – Real time polymerase chain reaction

SAH – S-adenosylhomocysteine

SAHH – S-adenosylhomocysteine hydrolase

SAM – S-adenosylmethionine

SEM – Standard error of the mean

SCG – Superior cervical ganglion

SIDS – Sudden infant death syndrome

SP – Substance P

TH – Tyrosine hydroxylase

TTX – Tetradotoxin

UDP - uridine 5'-diphosphate

VAChT – Vesicular acetylcholine transporter

VAH – Ventilatory acclimatisation to hypoxia

VEGF – Vascular endothelial growth factor

UV – Ultra violet

GLOSSARY

1 mmHg = 1 Torr

α-bungarotoxin – Selective antagonist of nicotinic ACh receptors with α7 subunits

AOPCP – Inhibitor of 5'-ectonucleotidase, inhibits ATP extracellular catabolism

Caffeine – Non-selective antagonist of adenosine receptors

CGS 21680 – Selective A_{2A} adenosine receptor agonist

Cytisine – Selective nicotinic ACh receptor agonist

DHßE – Selective nicotinic ACh receptor antagonist

Domperidone - Selective antagonist of D_2 dopamine receptors

DMPP - Selective nicotinic ACh receptor agonist

DPCPX – Agonist of A_1 adenosine receptors in nM concentrations, and also of A_{2B} adenosine receptors in µM concentrations

d-tubocurarine - Non selective nicotinic ACh receptor antagonist

Dipyridamole – Inhibitor of equilibrative nucleotide transport system

EC_{50} - drug concentration that produces 50% of maximal effect

EHNA – Inhibitor of adenosine deaminase, inhibits adenosine deamination

Haloperidol – Selective antagonist of D_2 dopamine receptors

HE-NECA - Selective A_{2A} adenosine receptor agonist

IC_{50} - drug concentration that produces 50% of maximal inhibition

Ionomycin - Alters cell permeability, producing an increase in intracellular Ca^{2+}

Mecamylamine – Non-selective allosteric inhibitor of nicotinic ACh receptors

MRS 1754 – Selective A_{2B} adenosine receptor antagonist

NBTI - Inhibitor of equilibrative nucleotide transport system

NECA – Non-selective agonist of A_2 adenosine receptors

Nicotine - Selective nicotinic ACh receptor agonist

Propylnorapomorphine – Selective D_2 dopamine receptor agonist

SCH 58621 - Selective A_{2A} adenosine receptor antagonist

Sulpiride – D_2-like dopamine receptor antagonist

ZM 241385 – Selective antagonist of A_{2A} and A_{2B} adenosine receptors, with higher affinity for A2A adenosine receptors

ABSTRACT

Carotid bodies (CB) are peripheral chemoreceptor organs sensing changes in arterial blood O_2, CO_2 and pH levels. Hypoxia and acidosis or hypercapnia activates CB chemoreceptor cells, which respond by releasing neurotransmitters in order to increase the action potential frequency in their sensory nerve, the carotid sinus nerve (CSN). CSN activity is integrated in the brainstem to induce a fan of cardiorespiratory reflex responses, aimed at normalising the altered blood gases. Exogenously applied adenosine (Ado) increases CSN chemosensory activity inducing hyperventilation through activation of A_2 receptors. The importance of the effects of adenosine in chemoreception was reinforced by data obtained in humans, in which the intravenous infusion of Ado causes hyperventilation and dyspnoea, an effect that has been attributed to the activation of CB because Ado does not cross blood-brain barrier and because the ventilatory effects are higher the closer to the CB it is injected.

The present work was performed in order to establish the functional significance of adenosine in chemoreception at the carotid body in control and chronically hypoxic rats. To achieve this objective we investigated: 1) The release of adenosine from a rat carotid body *in vitro* preparation in response to moderate hypoxia and the specificity of this release. We also investigated the metabolic pathways of adenosine production and release in the organ in normoxia and hypoxia; 2) The modulation of adenosine/ATP release from rat carotid body chemoreceptor cells by nicotinic ACh receptors; 3) The effects of caffeine on peripheral control of breathing and the identity of the adenosine receptors involved in adenosine and caffeine effects on carotid body chemoreceptors; 4) The interactions between dopamine D_2 receptors and adenosine A_{2B} receptors that modulate the release of catecholamines (CA) from the rat carotid body; 5) The effect of chronic caffeine intake i.e. the continuous blockage of adenosine receptors thereby simulating a caffeine dependence, on the carotid body function in control and chronically hypoxic rats. The methodologies used in this work included: molecular biology techniques (e.g. immunocytochemistry and western-blot), biochemical techniques (e.g. neurotransmitter quantification by HPLC, bioluminescence and radioisotopic

methods), electrophysiological techniques (e.g. action potential recordings) and ventilatory recordings using whole-body plethysmography.

It was observed that: 1) CB chemoreceptor sensitivity to hypoxia could be related to its low threshold for the release of adenosine because moderate acute hypoxia (10% O_2) increased adenosine concentrations released from the CB by 44% but was not a strong enough stimulus to evoke adenosine release from superior cervical ganglia and arterial tissue; 2) Acetylcholine (ACh) modulates the release of adenosine/5'-adenosine triphosphate (ATP) from CB in moderate hypoxia through the activation of nicotinic receptors with α_4 and ß$_2$ receptor subunits, suggesting that the excitatory role of ACh in chemosensory activity includes indirect activation of purinergic receptors by adenosine and ATP, which strongly supports the hypothesis that ATP/adenosine are important mediators in chemotransduction; 3) adenosine increases the release of CA from rat CB chemoreceptor cells via A_{2B} receptors; 4) the inhibitory effects of caffeine on CB chemoreceptors are mediated by antagonism of postsynaptic A_{2A} and presynaptic A_{2B} adenosine receptors indicating that chemosensory activity elicited by hypoxia is controlled by adenosine; 5) The release of CA from rat CB chemoreceptor cells is modulated by adenosine through an antagonistic interaction between A_{2B} and D_2 receptors, for the first time herein described; 6) chronic caffeine treatment did not significantly alter the basal function of CB in normoxic rats assessed as the dynamics of their neurotransmitters, dopamine, ATP and adenosine, and the CSN chemosensory activity. In contrast, the responses to hypoxia in these animals were facilitated by chronic caffeine intake because it increased the ventilatory response, slightly increased CSN chemosensory activity and increased dopamine (DA) and ATP release; 7) In comparison with normoxic rats, chronically hypoxic rats exhibited an increase in several parameters: ventilatory hypoxic response; basal and hypoxic CSN activity; tyrosine hydroxylase expression, CA content, synthesis and release; basal and hypoxic adenosine release; and in contrast a normal basal release and diminished hypoxia-induced ATP release; 8) Finally, in contrast to chronically hypoxic rats, chronic caffeine treatment did not alter the basal CSN chemosensory activity. Nevertheless, the responses to mild and intense hypoxia, and hypercapnia, were diminished. This inhibitory effect of chronic caffeine in CB output is compensated by central mechanisms, as the minute

ventilation parameter in basal conditions and in response to acute hypoxic challenges remained unaltered in rats exposed to chronic hypoxia.

We can conclude that adenosine both in acute and chronically hypoxic conditions have an excitatory role in the CB chemosensory activity, acting directly on adenosine A_{2A} receptors present postsynaptically in CSN, and acting presynaptically via A_{2B} receptors controlling the release of dopamine in chemoreceptor cells. We suggest that A_{2B} -D_2 adenosine / dopamine interactions at the CB could explain the increase in CA metabolism caused by chronic ingestion of caffeine during chronic hypoxia. It was also concluded that adenosine facilitates CB sensitisation to chronic hypoxia although this effect is further compensated at the central nervous system.

RESUMO

Os corpos carotídeos (CB) são pequenos orgãos emparelhados localizados na bifurcação da artéria carótida comum. Estes órgãos são sensíveis a variações na PaO_2, $PaCO_2$, pH e temperatura sendo responsáveis pela hiperventilação que ocorre em resposta à hipóxia, contribuindo também para a hiperventilação que acompanha a acidose metabólica e respiratória. As células quimiorreceptoras (tipo I ou glómicas) do corpo carotídeo respondem às variações de gases arteriais libertando neurotransmissores que activam as terminações sensitivas do nervo do seio carotídeo (CSN) conduzindo a informação ao centro respiratório central. Está ainda por esclarecer qual o neurotransmissor (ou os neurotransmissores) responsável pela sinalização hipóxica no corpo carotídeo. A adenosina é um neurotransmissor excitatório no CB que aumenta a actividade eléctrica do CSN induzindo a hiperventilação através da activação de receptores A_2. A importância destes efeitos da adenosina na quimiorrecepção, descritos em ratos e gatos, foi reforçada por resultados obtidos em voluntários saudáveis onde a infusão intravenosa de adenosina em induz hiperventilação e dispneia, efeito atribuído a uma activação do CB uma vez que a adenosina não atravessa a barreira hemato-encefálica e o efeito é quanto maior quanto mais perto do CB for a administração de adenosina.

O presente trabalho foi realizado com o objectivo de esclarecer qual o significado funcional da adenosina na quimiorrecepção no CB em animais controlo e em animais submetidos a hipoxia crónica mantida. Para alcançar este objectivo investigou-se: 1) o efeito da hipóxia moderada sobre a libertação de adenosina numa preparação *in vitro* de CB e a especificidade desta mesma libertação comparativamente com outros tecidos não quimiossensitivos, assim como as vias metabólicas de produção e libertação de adenosina no CB em normoxia e hipóxia; 2) a modulação da libertação de adenosina/ATP das células quimiorreceptoras do CB por receptores nicotínicos de ACh; 3) os efeitos da cafeína no controlo periférico da ventilação e a identidade dos receptores de adenosina envolvidos nos efeitos da adenosina e da cafeína nos quimiorreceptores do CB; 4) as interacções entre os receptores D_2 de dopamina e os receptores A_{2B} de adenosina que modulam a libertação de

catecolaminas (CA) no CB de rato e; 5) o efeito da ingestão crónica de cafeína, isto é, o contínuo bloqueio e dos receptores de adenosina, simulando assim o consumo crónico da cafeína, tal como ocorre na população humana mundial e principalmente no ocidente, na função do corpo carotídeo em ratos controlo e em ratos submetidos a hipoxia crónica.

Os métodos utilizados neste trabalho incluíram: técnicas de biologia molecular como imunocitoquímica e western-blot; técnicas bioquímicas, tais como a quantificação de neurotransmissores por HPLC, bioluminescência e métodos radioisotópicos; técnicas electrofisiológicas como o registro de potenciais eléctricos do nervo do seio carotídeo *in vitro*; e registros ventilatórios *in vivo* em animais não anestesiados e em livre movimento (pletismografia).

Observou-se que: **1)** a especificidade dos quimiorreceptores do CB como sensores de O_2 está correlacionada com o baixo limiar de libertação de adenosina em resposta à hipóxia dado que a libertação de adenosina do CB aumenta 44% em resposta a uma hipóxia moderada (10% O_2), que no entanto não é um estímulo suficientemente intenso para evocar a libertação de adenosina do gânglio cervical superior ou do tecido arterial. Observou-se também que aproximadamente 40% da adenosina libertada pelo CB provém do catabolismo extracelular do ATP quer em normóxia quer em hipóxia moderada, sendo que PO_2 reduzidas induzem a libertação de adenosina via activação do sistema de transporte equilibrativo ENT1.

2) a ACh modula a libertação de adenosina /ATP do CB em resposta à hipoxia moderada sugerindo que o papel excitatório da ACh na actividade quimiossensora inclui a activação indirecta de receptores purinérgicos pela adenosina e ATP, indicando que a adenosina e o ATP poderiam actuar como mediadores importantes no processo de quimiotransducção uma vez que: a) a activação dos receptores nicotínicos de ACh no CB em normóxia estimula a libertação de adenosina (max 36%) provindo aparentemente da degradação extracelular do ATP. b) a caracterização farmacológica dos receptores nicotínicos de ACh envolvidos na estimulação da libertação de adenosina do CB revelou que os receptores nicotínicos de ACh envolvidos são constituídos por subunidades α4ß2.

3) a adenosina modula a libertação de catecolaminas das células quimiorreceptoras do CB através de receptores de adenosina A_{2B} dado que: a)

a cafeína, um antagonista não selectivo dos receptores de adenosina, inibiu a libertação de CA quer em normóxia quer em resposta a estímulos de baixa intensidade sendo ineficaz na libertação induzida por estímulos de intensidade superior; b) o DPCPX e do MRS1754 mimetizaram os efeitos da cafeína no CB sendo o SCH58621 incapaz de induzir a libertação de CA indicando que os efeitos da cafeína seriam mediados por receptores A_{2B} de adenosina cuja presença nas células quimiorreceptoras do CB demonstramos por imunocitoquímica.

4) a aplicação aguda de cafeína inibiu em 52% a actividade quimiossensora do CSN induzida pela hipóxia sendo este efeito mediado respectivamente por receptores de adenosina A_{2A} pós-sinápticos e A_{2B} pré-sinápticos indicando que a actividade quimiossensora induzida pela hipóxia é controlada pela adenosina.

5) existe uma interacção entre os receptores A_{2B} e D_2 que controla a libertação de CA do corpo carotídeo de rato uma vez que: a) os antagonistas dos receptores D_2, domperidona e haloperidol, aumentaram a libertação basal e evocada de CA das células quimiorreceptoras confirmando a presença de autorreceptores D_2 no CB de rato que controlam a libertação de CA através de um mecanismo de feed-back negativo. b) o sulpiride, um antagonista dos receptores D_2, aumentou a libertação de CA das células quimiorreceptoras revertendo o efeito inibitório da cafeína sobre esta mesma libertação; c) a propilnorapomorfina, um agonista D_2 inibiu a libertação basal e evocada de CA sendo este efeito revertido pela NECA, um agonista dos receptores A_{2B}. O facto de a NECA potenciar o efeito do haloperidol na libertação de CA sugere que a interacção entre os receptores D_2 e A_{2B} poderia também ocorrer ao nível de segundos mensageiros, como o cAMP.

6) a ingestão crónica de cafeína em ratos controlo (normóxicos) não alterou significativamente a função basal do CB medida como a dinâmica dos seus neurotransmissores, dopamina, ATP e adenosina e como actividade quimiossensora do CSN. Contrariamente aos efeitos basais, a ingestão crónica de cafeína facilitou a resposta à hipóxia, dado que aumentou o efeito no volume minuto respiratórioapresentando-se também uma clara tendência para aumentar a actividade quimiossensora do CSN e aumentar a libertação de ATP e dopamina.

7) após um período de 15 dias de hipóxia crónica era evidente o fenómeno de aclimatização dado que as respostas ventilatórias à hipóxia se encontram aumentadas, assim como a actividade quimiossensora do CSN basal e induzida pela hipóxia. As alterações observadas no metabolismo da dopamina, assim como na libertação basal de dopamina e de adenosina poderiam contribuir para a aclimatização durante a hipoxia crónica. A libertação aumentada de adenosina em resposta à hipóxia aguda em ratos hipóxicos crónicos sugere um papel da adenosina na manutenção/aumento das respostas ventilatórias à hipóxia aguda durante a hipóxia crónica. Observou-se também que a libertação de ATP induzida pela hipóxia aguda se encontra diminuída em hipóxia crónica, contudo a ingestão crónica de cafeína reverteu este efeito para valores similares aos valores controlo, sugerindo que a adenosina possa modular a libertação de ATP em hipóxia crónica.

8) a ingestão crónica de cafeína em ratos hipóxicos crónicos induziu o aumento do metabolismo de CA no CB, medido como expressão de tirosina hidroxilase, conteúdo, síntese e libertação de CA.

9) a ingestão crónica de cafeína não provocou quaisquer alterações na actividade quimiossensora do CSN em ratos hipóxicos crónicos no entanto, as respostas do CSN à hipóxia aguda intensa e moderada e à hipercapnia encontram-se diminuídas. Este efeito inibitório que provém da ingestão crónica de cafeína parece ser compensado ao nível dos quimiorreceptores centrais dado que os parâmetros ventilatórios em condições basais e em resposta à hipoxia aguda não se encontram modificados em ratos expostos durante 15 dias a uma atmosfera hipóxica.

Resumindo podemos assim concluir que a adenosina quer em situações de hipoxia aguda quer em condições de hipoxia crónica tem um papel excitatório na actividade quimiossensora do CB actuando directamente nos receptores A_{2A} presentes pós-sinapticamente no CSN, assim como facilitando a libertação de dopamina pré-sinapticamente via receptores A_{2B} presentes nas células quimiorreceptoras. A interacção negativa entre os receptores A_{2B} e D_2 observadas nas células quimiorreceptoras do CB poderia explicar o aumento do metabolismo de CA observado após a ingestão crónica de cafeína em animais hipóxicos. Conclui-se ainda que durante a aclimatização à hipóxia a acção inibitória da cafeína, em termos de resposta ventilatória, mediada pelos

quimiorreceptores periféricos é compensada pelos efeitos excitatórios desta xantina ao nível do quimiorreceptores centrais.

RESUMEN

Los cuerpos carotídeos (CB) son órganos emparejados que están localizados en la bifurcación de la arteria carótida común. Estos órganos son sensibles a variaciones en la PaO_2, en la $PaCO_2$, pH y temperatura siendo responsables de la hiperventilación que ocurre en respuesta a la hipoxia, contribuyendo también a la hiperventilación que acompaña a la acidosis metabólica y respiratoria. Las células quimiorreceptoras (tipo I o glómicas) del cuerpo carotídeo responden a las variaciones de gases arteriales liberando neurotransmissores que activan las terminaciones sensitivas del nervio del seno carotídeo (CSN) llevando la información al centro respiratorio central. Todavía esta por clarificar cual el neurotransmisor (o neurotransmisores) responsable por la señalización hipóxica en el CB. La adenosina es un neurotransmisor excitatório en el CB ya que aumenta la actividad del CSN e induce la hiperventilación a través de la activación de receptores de adenosina del subtipo A_2. La importancia de estos efectos de la adenosina en la quimiorrecepción, descritos en ratas y gatos, ha sido fuertemente reforzada por resultados obtenidos en voluntarios sanos en los que la infusión intravenosa de adenosina induce hiperventilación y dispnea, efectos estés que han sido atribuidos a una activación del CB ya que la adenosina no cruza la barrera hemato-encefalica y el efecto es tanto más grande cuanto más cercana del CB es la administración.

Este trabajo ha sido realizado con el objetivo de investigar cual el significado funcional de la adenosina en la quimiorrecepción en el CB en animales controlo y en animales sometidos a hipoxia crónica sostenida. Para alcanzar este objetivo se ha estudiado: 1) el efecto de la hipoxia moderada en la liberación de adenosina en una preparación *in vitro* de CB y la especificidad de esta liberación en comparación con otros tejidos no-quimiosensitivos, así como las vías metabólicas de producción y liberación de adenosina del órgano en normoxia y hipoxia; 2) la modulación de la liberación de adenosina/ATP de las células quimiorreceptoras del CB por receptores nicotínicos de ACh; 3) los efectos de la cafeína en el controlo periférico de la ventilación y la identidad de los receptores de adenosina involucrados en los efectos de la adenosina y

cafeína en los quimiorreceptores del CB; 4) las interacciones entre los receptores D_2 de dopamina y los receptores A_{2B} de adenosina que modulan la liberación de catecolaminas (CA) en el CB de rata y; 5) el efecto de la ingestión crónica de cafeína, es decir, el bloqueo sostenido de los receptores de adenosina, simulando la dependencia de cafeína observada en la población mundial del occidente, en la función del CB en ratas controlo y sometidas a hipoxia crónica sostenida.

Los métodos utilizados en este trabajo incluirán: técnicas de biología molecular como imunocitoquímica y western-blot; técnicas bioquímicas, tales como la cuantificación de neurotransmissores por HPLC, bioluminescencia y métodos radioisotópicos; técnicas electrofisiológicas como el registro de potenciales eléctricos del nervio do seno carotídeo *in vitro*; y registros ventilatórios *in vivo* en animales no anestesiados y en libre movimiento (pletismografia).

Se observó que: 1) la sensibilidad de los quimiorreceptores de CB esta correlacionada con un bajo umbral de liberación de adenosina en respuesta a la hipoxia ya que en respuesta a una hipoxia moderada (10% O_2) la liberación de adenosina en el CB aumenta un 44%, sin embargo esta PaO_2 no es un estimulo suficientemente fuerte para inducir la liberación de adenosina del ganglio cervical superior o del tejido arterial; se observó también que aproximadamente 40% de la adenosina liberada del CB proviene del catabolismo extracelular del ATP en normoxia y en hipoxia moderada, y que bajas PO_2 inducen la liberación de adenosina vía activación del sistema de transporte equilibrativo ENT1.

2) la ACh modula la liberación de adenosina /ATP del CB en respuesta a la hipóxia moderada lo que sugiere que el papel excitatório de la ACh en la actividad quimiosensora incluye la activación indirecta de receptores purinérgicos por la adenosina y el ATP, indicando que la adenosina y el ATP pueden actuar como mediadores importantes en el proceso de quimiotransducción ya que: a) la activación de los receptores nicotínicos de ACh en el CB en normoxia estimula la liberación de adenosina (max 36%) que aparentemente proviene de la degradación extracelular del ATP. Se observó también que este aumento de adenosina en el CB en hipoxia ha sido antagonizado parcialmente por antagonistas de estos mismos receptores; b) la

caracterización farmacológica de los receptores nicotínicos de ACh involucrados en la estimulación de la liberación de adenosina del CB ha revelado que los receptores nicotínicos de ACh involucrados son constituidos por sub-unidades α4ß2.

3) la adenosina modula la liberación de CA de las células quimiorreceptoras del CB a través de receptores de adenosina A_{2B} ya que: a) la cafeína, un antagonista no selectivo de los receptores de adenosina, ha inhibido la liberación de CA en normoxia y en respuesta a estímulos de baja intensidad siendo ineficaz en la liberación inducida por estímulos de intensidad superior; b) el DPCPX y el MRS1754 ha mimetizado los efectos de la cafeína en el CB y el SCH58621 ha sido incapaz de inducir la liberación de CA lo que sugiere que los efectos de la cafeína son mediados por receptores A_{2B} de adenosina que están localizados pré-sinapticamente en las células quimiorreceptoras del CB.

4) la aplicación aguda de cafeína ha inhibido en 52% la actividad quimiosensora del CSN inducida por la hipoxia siendo este efecto mediado respectivamente por receptores de adenosina A_{2A} pós-sinápticos y A_{2B} pré-sinápticos lo que indica que la actividad quimiosensora inducida por la hipoxia es controlada por la adenosina.

5) existe una interacción entre los receptores A_{2B} y D_2 que controla la liberación de CA del CB de rata ya que: a) el sulpiride, un antagonista de los receptores D_2, ha aumentado la liberación de CA de las células quimiorreceptoras revertiendo el efecto inhibitorio de la cafeína sobre esta misma liberación; b) los antagonistas de los receptores D_2, domperidona y haloperidol, han aumentado la liberación basal e evocada de CA de las células quimiorreceptoras confirmando la presencia de autorreceptores D_2 en el CB de rata que controlan la liberación de CA a través de un mecanismo de feed-back negativo; c) la propilnorapomorfina, un agonista D_2, ha inhibido la liberación basal e evocada de CA sendo este efecto revertido por la NECA, un agonista de los receptores A_{2B}. Ya que la NECA potencia el efecto del haloperidol en la liberación de CA la interacción entre los D_2 y A_{2B} puede también ocurrir al nivel de segundos mensajeros, como el cAMP.

6) la ingestión crónica de cafeína en ratas controlo (normóxicas) no ha cambiado significativamente la función basal del CB medida como la dinámica de sus neurotransmisores, dopamina, ATP y adenosina y como actividad

quimiosensora del CSN. Al revés de lo que pasa con los efectos básales, la ingestión crónica de cafeína facilitó la respuesta a la hipóxia, ya que ha aumentado la respuesta ventilatória medida como volumen minuto presentando también una clara tendencia para aumentar la actividad quimiosensora del CSN y aumentar la liberación de ATP y dopamina.

7. Después de un período de 15 días de hipoxia crónica se puede observar el fenómeno de climatización ya que las respuestas ventilatórias a la hipoxia están aumentadas, así como la actividad quimiosensora del CSN basal e inducida por la hipoxia. Los cambios observados en el metabolismo de la dopamina, así como en la liberación basal de dopamina y de adenosina podrían contribuir para la climatización en hipoxia crónica. El aumento en la liberación de adenosina en respuesta a la hipoxia aguda en ratas sometidas a hipoxia crónica sugiere un papel para la adenosina en el mantenimiento/aumento de las respuestas ventilatórias a la hipoxia aguda en hipoxia crónica sostenida. Se ha observado también que la liberación de ATP inducida por la hipoxia aguda está disminuida en hipoxia crónica y que la ingestión crónica de cafeína reverte este efecto para valores similares a los valores controlo, sugiriendo que la adenosina podría modular la liberación de ATP en hipoxia crónica.

8. la ingestión crónica de cafeína ha inducido el aumento del metabolismo de CA en el CB en ratas hipóxicas crónicas, medido como expresión de la tirosina hidroxilase, contenido, síntesis y liberación de CA.

9. la ingestión crónica de cafeína no ha inducido cambios en la actividad quimiosensora del CSN en ratas hipóxicas crónicas sin embargo las respuestas do CSN a una hipoxia intensa y moderada y a la hipercapnia están disminuidas. Este efecto inhibitorio que es debido a la ingestión crónica de cafeína es compensado al nivel de los quimiorreceptores centrales ya que los parámetros ventilatórios en condiciones básales y en respuesta a la hipoxia aguda no están modificados en ratas expuestas durante 15 días a una atmósfera hipóxica.

Resumiendo se puede concluir que la adenosina en situaciones de hipoxia aguda así como en hipoxia crónica tiene un papel excitatório en la actividad quimiosensora del CB actuando directamente en los receptores A_{2A} localizados pós-sinapticamente en el CSN, así como controlando la liberación

de dopamina pré-sinaptica vía receptores A_{2B} localizados en las células quimiorreceptoras. Las interacciones entre los receptores A_{2B} y D_2 observadas en las células quimiorreceptoras del CB podrían explicar el aumento del metabolismo de CA observado después de la ingestión crónica de cafeína en animales hipóxicos. Por fin, pero no menos importante se puede concluir que durante la climatización a la hipoxia la acción inhibitoria de la cafeína, medida como respuesta ventilatória, mediada por los quimiorreceptores periféricos es compensada por los efectos excitatórios de esta xantina al nivel de los quimiorreceptores centrales.

1. GENERAL INTRODUCTION

Oxygen is used in cells by cellular respiration to oxidize nutrients and to obtain the energy (ATP) required for cell function, originating CO_2 and water as final products. The capture of oxygen from the atmosphere is done by the respiratory and circulatory systems. The process of breathing allows the continuous delivery of O_2-rich fresh air into the lungs and the alveoli. When atmospheric air enters into the alveoli an exchange of gases is produced. The exchange of gases (O_2 and CO_2) between the alveoli and the blood occurs by simple diffusion: O_2 diffusing from the alveoli into the blood and CO_2 from the blood into the alveoli. Since diffusion requires a concentration gradient, the concentration (or pressure) of O_2 in the alveoli must be kept at a higher level than in the blood and the concentration (or pressure) of CO_2 in the alveoli must be kept at a lower level than in the blood. This gaseous exchange and mixing results in a partial arterial pressure of oxygen (PaO_2) of 100 mmHg and a partial arterial pressure of carbon dioxide ($PaCO_2$) of 35-45 mmHg.

If O_2 delivery to the cells is insufficient to perform all cell functions i.e. when cells do not have enough oxygen to function properly, we are in a situation of hypoxia. In its extreme form, when O_2 is entirely absent, the condition is called anoxia. There are four types of hypoxia: hypoxic hypoxia, which is the most common and is caused by decreased oxygen in air or by the inability of oxygen to diffuse across the lungs walls; hypoxemic (anaemic) hypoxia that is caused by the reduction of the oxygen carrying capacity of the blood (low haemoglobin); ischemic (stagnant) hypoxia that is caused by reduced cardiac output and resultant slower blood circulation rate; and histotoxic hypoxia that occurs when cells are incapable of using O_2. Physiologically, only hypoxic hypoxia could occur in a healthy animal, as all the other hypoxias would be pathological. Pathologically, several cardiorespiratory diseases result in chronic sustained or intermittent hypoxia. These include e.g. sleep apnoea, congestive heart failure, emphysema, chronic obstructive pulmonary disease (COPD) and sudden infant death syndrome (SIDS). Chronic exposure to hypoxia regulates the expression of numerous genes, encoding enzymes, growth factors, or transporters, which induce molecular and

histological modifications to reduce the cellular need of, and dependence on, O_2 and increase the O_2 supply to the tissues (figure 1).

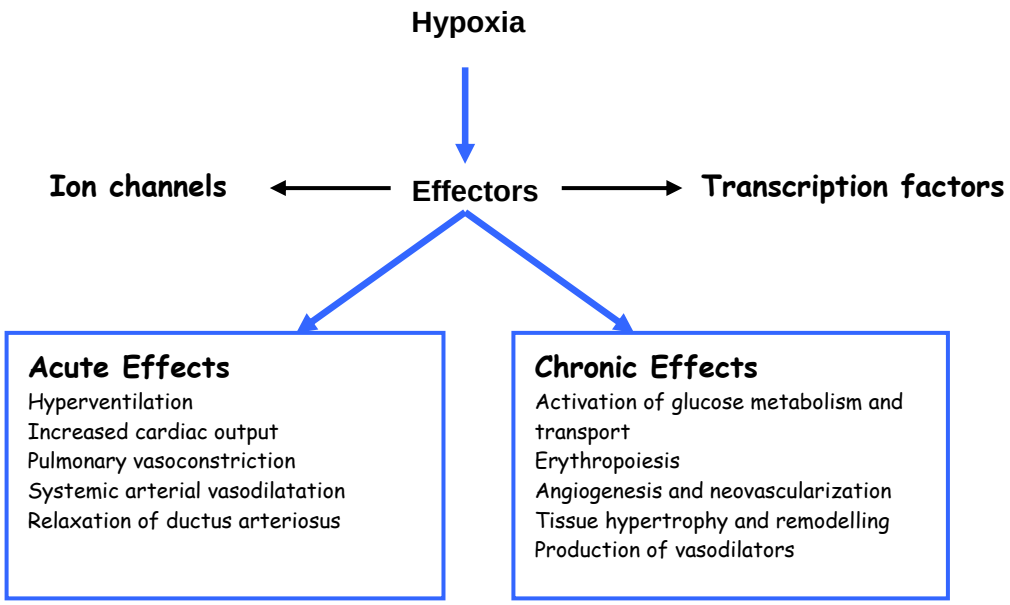


Figure 1 Hypoxia signalling pathway with the major adaptive responses to acute and chronic hypoxia (adapted from Lopez-Barneo et al., 2004).

Mammals possess regulatory mechanisms that, acting on the respiratory and cardiovascular systems, try to minimize hypoxia and to prevent its deleterious effects. The arterial chemoreceptors, aortic bodies and in particular the carotid bodies (CB) are the activators of those regulatory mechanisms. The CBs are located near the carotid artery bifurcations and sense arterial PO_2 (PaO_2), arterial PCO_2 ($PaCO_2$), pH and temperature, being responsible for the greatest part of the hyperventilation observed during hypoxia and contributing to the hyperventilation that accompanies respiratory or metabolic acidosis. The remaining respiratory drive is due to aortic bodies in the case of hypoxia and to central chemoreceptors in the case of acidosis (Richerson and Boron, 2005).

CB chemoreceptor cells are sensors that, in response to low PO_2, high PCO_2 and pH, release neurotransmitters that modify, increasing or inhibiting, the frequency of sensory fibres of the carotid sinus nerve (CSN). Central projections of the CSN terminate in the brain stem, where the firing frequency is integrated by the respiratory central control system, generating the compensatory ventilatory response.

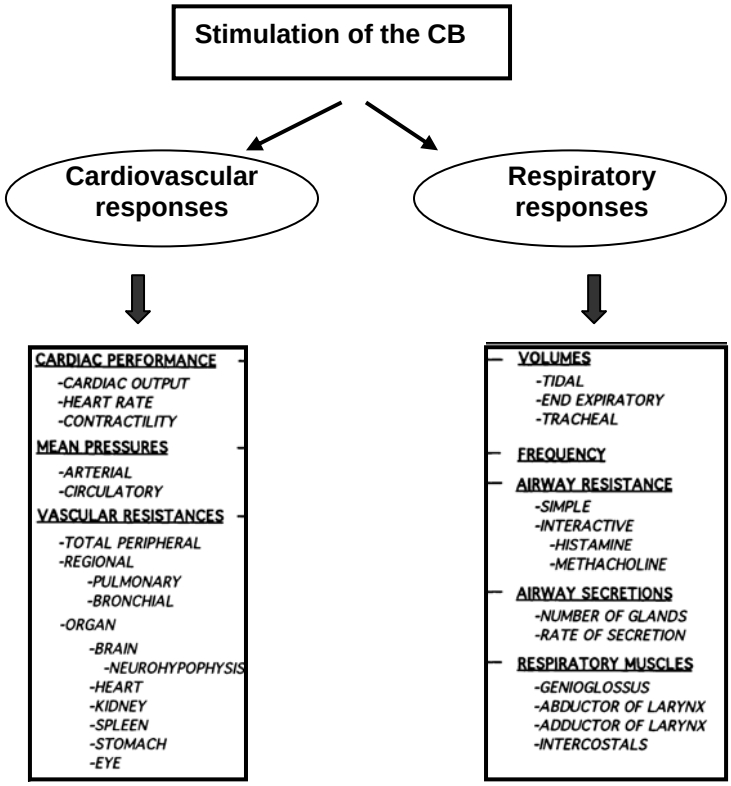


Figure 2 Respiratory and cardiovascular responses to carotid body activation. Adapted from Fitzgerald, 2000.

Chemoreceptors also exert some actions on the cardiovascular system, namely the heart and the resistance and capacitance of vessels (Figure 2) (for a review see Gonzalez et al., 1994).

The CBs have been known as sensory organs since 1928 (De Castro, 1928) and in 1938 the Nobel Prize was awarded to Heymans for the discovery of “the aortic and sinus mechanisms of respiration”. Over the subsequent decades the CB was extensively studied in terms of reflex systemic responses resulting from CB stimulation and in terms of the physiology and pharmacology of neural mechanisms responsible for the action potentials generated at CSN level and integrated at the brain stem, producing systemic responses. Several theories were postulated, aimed at explaining the chemoreception process, the “mechanism and the substances involved” that contribute to the stimulus-generated neural activity in the CSN.

Adenosine is a mediator in the central and peripheral nervous system and it is known that it stimulates ventilation in several mammalian species, including humans. In the recent years an excitatory role for adenosine has been

proposed at the CB and in this context we decided to investigate the functional significance of this mediator in carotid body chemosensory activity, in control and chronically hypoxic animals.

1.1. The carotid body: morphology

The CB parenchyma is characterized by the existence of islets of cells, called clusters, glomeruli or glomoids enveloped by an extensive net of fenestrated capillaries and dispersed in a stroma of fibrocytes and collagen fibres (Figure 3C) (for a review see e.g. Verna, 1997). Each glomerulus contains two cell types: glomic cells (Type I cells or chemoreceptor cells) and Type II cells or sustentacular cells, and the surrounding capillary networks (Figure 3).

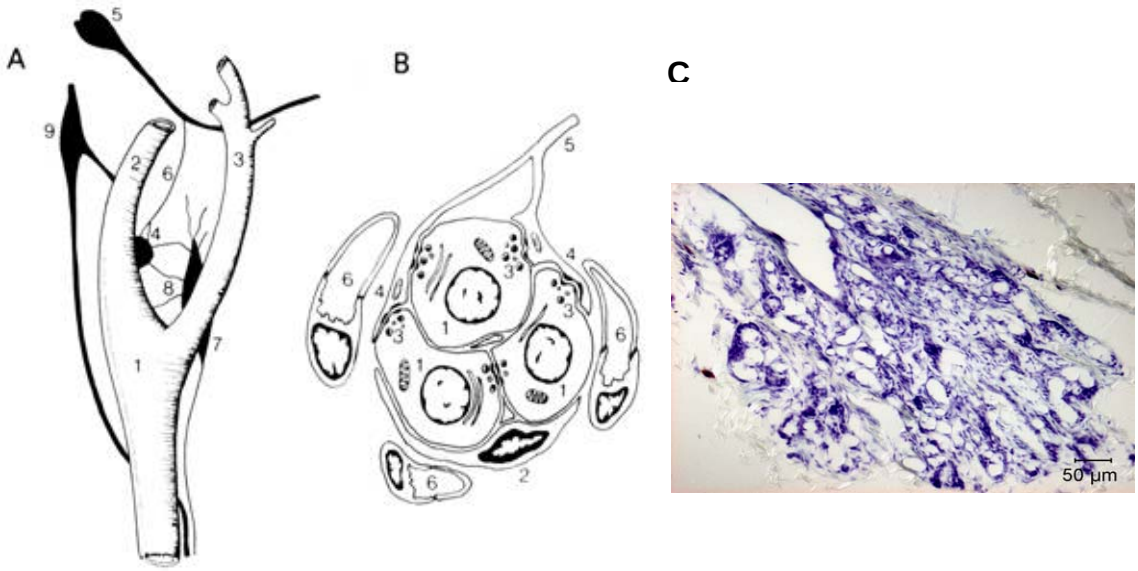


Figure 3 Drawing of the carotid artery bifurcation (**A**) and a cellular cluster of the carotid body (**B**); and a histological image of a slice of 10 µM of the carotid body (**C**). In **A** the common carotid artery (1) that divides to internal (2) and external (3) arteries. Sensory fibres originated in petrosal ganglion (5) reach the carotid body (4) via carotid sinus nerve (CSN) (6). The superior cervical ganglion (7) also innervates the carotid body, via the ganglioglomerular nerves (8).The nodose ganglion (9) is located externally to the internal carotid artery. **B** Cluster of parenchymatous cells of the carotid body, which are formed by chemoreceptor cells (1) that are partially surrounded by sustencular cells (2). Sensory nerve endings (4) are in contact with chemoreceptor cells. The clusters are surrounded by a dense net of capillaries (6). **C** Immunohistochemical image of a 10 µM section of the CB. (Adapted from Gonzalez et al., 1992)

Ultrastructural studies have demonstrated that chemoreceptor cells have characteristics of active secretory cells (Verna, 1979). Chemoreceptor cells are derived from the neural crest and possess a heterogeneous population of synaptic vesicles in their cytoplasm that contain some of the putative neurotransmitters. The neuroactive agents present in the chemoreceptors include acetylcholine (ACh), dopamine (DA), norepinephrine (NE) and serotonin (5-HT) and also several neuropeptides, like substance P (SP) and met-encephalin (ME) and ATP (Gonzalez et al., 1994; Zhang et al., 2000; Buttigieg and Nurse, 2004; Conde and Monteiro, 2006). Type II cells have a glial nature and lack specialized organelles (Gonzalez et al., 1992) (Figure 3B).

The proximity of capillaries to chemoreceptor cells (less than 20 μM) minimizes the diffusion pathway of bloodborn stimuli.

The CSN sensory fibres come from the petrosal ganglion (PG) neurons and penetrate the glomeruli, ending in synaptic apposition to the chemoreceptor cells (Figure 3B). Beyond this sensory innervation, the CB receives sympathetic and parasympathetic innervation in the vessels that is originated in the neurons present in the superior cervical ganglion (SCG) and at the surface of the CB and in other neurons dispersed through CSN. These fibres are noradrenergic and cholinergic and control the blood rate to the CB, i.e. its functional activity (Verna, 1997).

1.2. Chemotransduction mechanisms at the CB: coupling stimulation and secretion

It is universally accepted that chemoreceptor cells are the initial (first) transducers of sensorial stimulus releasing neurotransmitters that generate action potentials in CSN sensitive nerve fibres, this activity being integrated at the brain stem to induce ventilation (Figure 4).

At normal blood gas pressures and pH, chemoreceptor cells possess a basal activity that can be measured as basal release of neurotransmitters or as basal CSN electrical activity. An important role has been attributed to CB chemoreceptors in maintaining the resting ventilation since CB resection or CSN denervation produces alterations in the different resting respiratory

parameters, like a decrease in minute ventilation and a moderate increase of $PaCO_2$ (8 - 10 mmHg) (Bisgard et al., 1976; Bisgard and Vogel, 1971; Eugenin et al., 1989; Feustel et al., 1981). The PaO_2 threshold to hypoxia corresponds to 70-75 mmHg (Biscoe et al. 1970; Obeso et al.; 1997b) and below this PaO_2 the slope between PO_2 and CSN discharges and minute ventilation increase exponentially (for a review see Gonzalez et al., 1994, Figure 6).

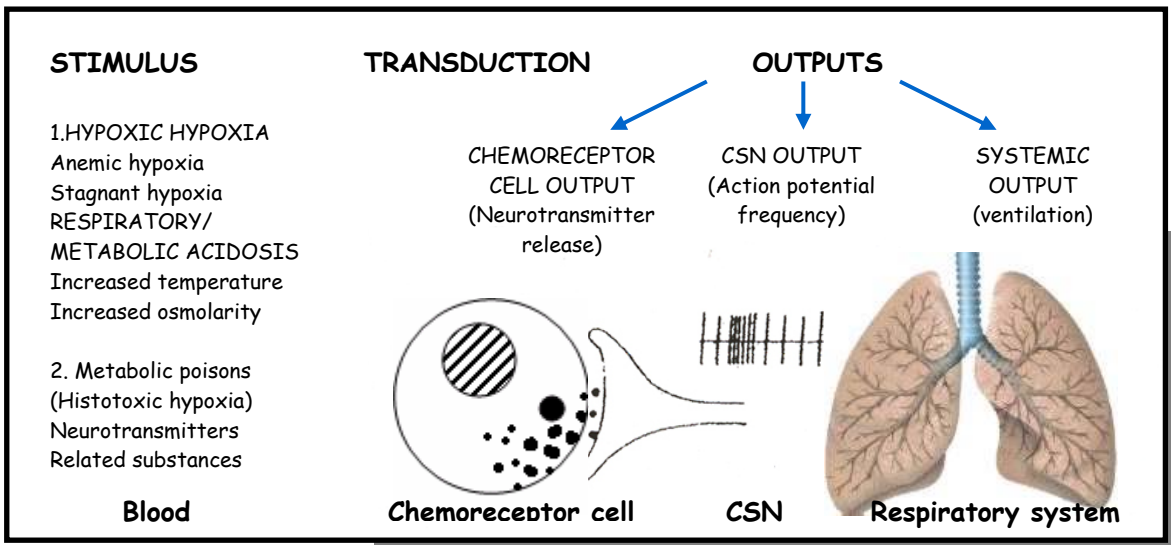


Figure 4 Functional organization of carotid body (CB) chemoreceptors. (Adapted from Gonzalez et al., 1994)

However, for the hypercapnic stimulus the relation between the increase in $PaCO_2$ and CSN discharges and release of neurotransmitters is different, being linear between 20-100 mmHg (Rigual et al., 1991). These relationships suggest that the transduction mechanisms are different for the two stimuli.

Several studies have shown that DA is released in hypoxia and hypercapnia, this release being proportional to stimulus intensity and to CSN chemosensory activity increase and dependent on extracellular Ca^{2+} (Fidone et al., 1982; Obeso et al., 1985, 1986, 1992, 1999; Rigual et al., 1986, 1991, 2002; Rocher et al., 1991; Vicario et al., 2000a; Sanz-Alfayate, 2001). The same effect was observed with the response of CB to high K^+ (depolarizing stimulus) (Almaraz et al., 1986). The response to hypoxia and to high K^+ was sensitive to dihydropyridines (L-type agonists and antagonists – voltage Ca^{2+} channels

dependent), however the acidic-stimulus response was dihydropyridine insensitive (Obeso et al., 1992). Soon after, it was demonstrated that veratridine, activator of voltage-dependent Na^+ channels, increases the release of DA, the release being dependent on Na^+ and Ca^{2+} and sensitive to tetradotoxin (TTX); in addition it was shown that the release of DA induced by hypoxia was also TTX-sensitive. All these findings suggest that hypoxia depolarizes chemoreceptor cells inducing Na^+ and Ca^{2+} entrance.

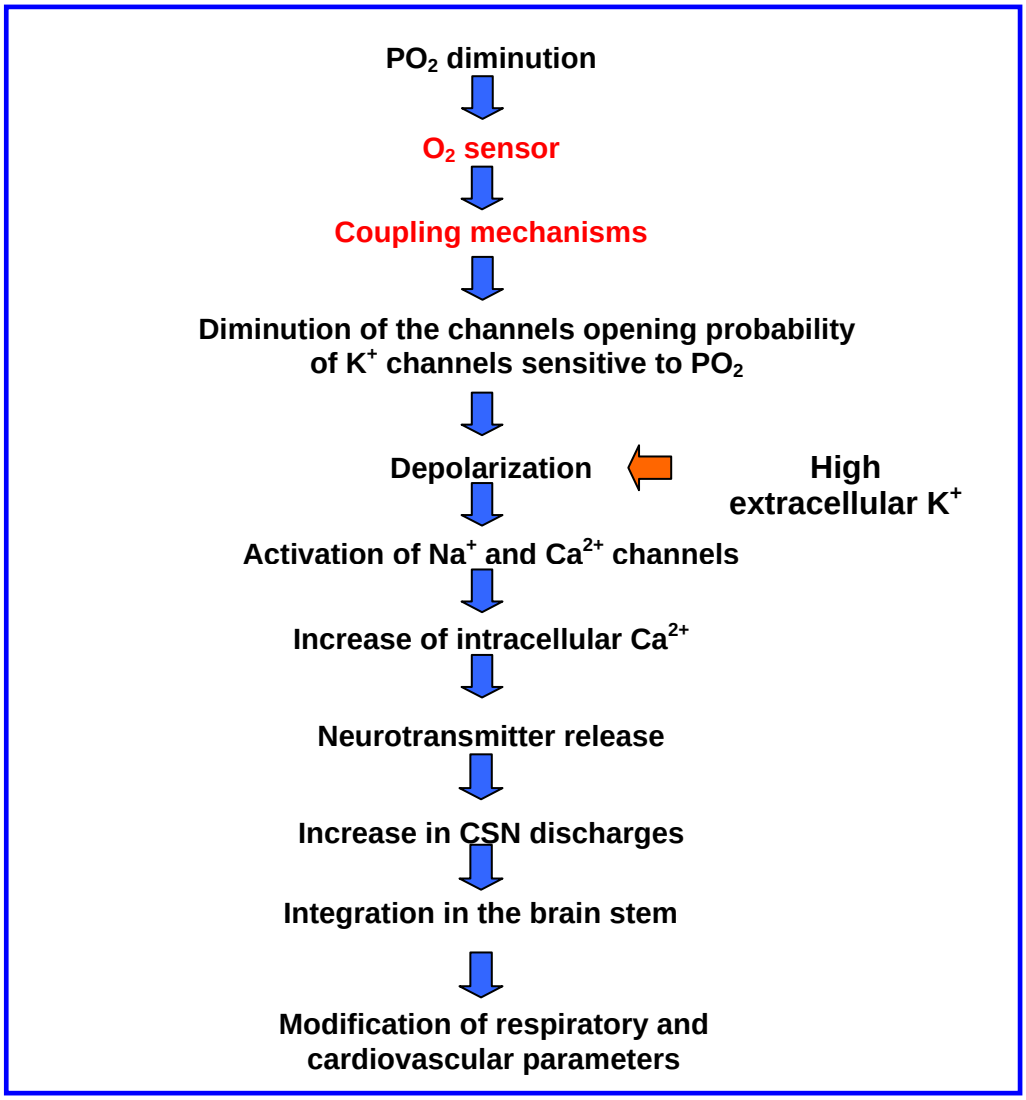


Figure 5 Hypoxic transduction mechanism at the carotid body. In red are the steps of the cascade not completely defined (established) (adapted from Gonzalez et al., 1992).

Next, K^+ currents sensitive to PO_2 that were inhibited by hypoxia were found in the CB chemoreceptor cells as well as voltage-dependent Na^+ and Ca^{2+} channels (Lopez-Barneo et al., 1988; Lopez-Lopez et al., 1989).

Taken together these findings led to the transduction model of hypoxia in the CB (Figure 5, Gonzalez et al., 1992). Nevertheless, some steps of transduction cascade are still to be defined, like the nature of the O_2 sensor and the coupling mechanisms.

1.3. Function and control of the carotid body in resting conditions and in hypoxia

In normal conditions of blood gases and pH, CSN fiber discharge varies with the type of fiber studied. Chemoreceptor A fibres discharge between 1-15 impulses/s and C-fibres discharge never more than 4-5 impulses/s, most of the time firing less than 2 impulses/s (Fidone and Sato, 1969).

Single nerve fiber activity is random because interspike intervals follow a Poison distribution; however, if resting discharges of a single nerve fiber are averaged over several respiratory cycles an oscillatory pattern becomes evident. The oscillations in CSN discharge have their origin in the periodic nature of breathing that produces oscillations in the alveolar PO_2 and PCO_2 that are transmitted to blood and detected by the CB.

The fact that in resting conditions the amplitude of the oscillations in relation to the mean CSN frequency is about 0.5, and the fact that CB resection or CSN denervation causes a decrease in resting minute ventilation and PaO_2, and an increase in $PaCO_2$, means that oscillations on CSN discharges are a sign as important as mean discharge (for a review see Gonzalez et al., 1994).

The data shown in figure 6A indicate that there is not a true threshold for the CB response to low PaO_2, because the CSN is already firing at PaO_2 5 times higher than the normal PaO_2 of 100 mmHg. However, it is usual to talk about the “physiological” threshold for CSN activation. The PaO_2 threshold to hypoxia corresponds to 70-75 mmHg (Biscoe et al. 1970; Obeso et al.; 1997b) and below this PaO_2 the slope between PO_2 and CSN discharges changes abruptly from linear to exponential until 10-15 mmHg (Gonzalez et al.,1994) (Figure 6A); at very low PO_2 (<10 mmHg) the discharges may tend to level off or to decrease. At a PaO_2 of around 75 mmHg the O_2 content of the blood is approximately 95% of the normal, which implies that CB triggers

hyperventilation before real tissular hypoxia appears, the CB having a tissue hypoxia-preventative role.

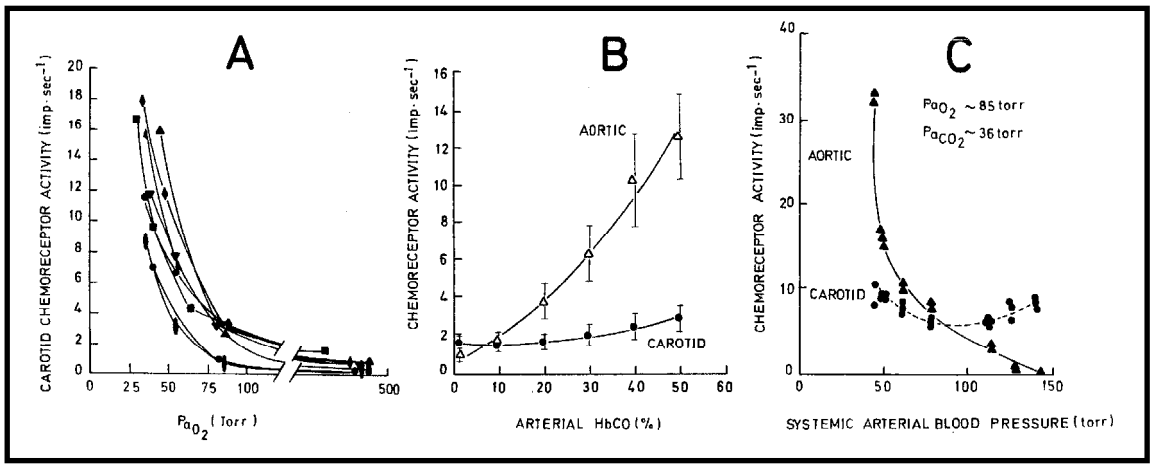


Figure 6 Carotid sinus nerve chemosensory activity during acute hypoxic stimulation. A) steady-state activity as a function of PaO_2 in different experiments (Source: Mullingan et al., 1981). B and C) Comparison between carotid body and aortic bodies' response in anemic hypoxia and stagnant hypoxia, respectively (Lahiri et al., 1980, 1981).

The relation between PaO_2 and ventilation is similar to that described for PaO_2 and CSN chemosensory activity (Weil et al., 1970; Figure 7). In humans in which CB have been surgically removed (common practice in the 1950´s and 60´s to treat severe asthma) the hyperventilatory response to hypoxia was not observed, these individuals presenting a ventilatory response to exercise that is reduced by 30% when compared with control subjects. Similar results have been found in animals: the response to hypoxia is abolished with CSN section (Lahiri, 1976, Figure 7).

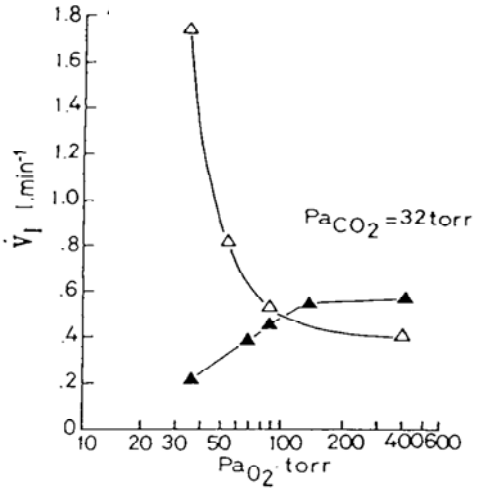


Figure 7 Effect of several hypoxic intensities on minute ventilation (VI; l/min) in a Nembutal-anesthetized cat before (∆) and after sectioning both carotid sinus nerves (▲). (Lahiri, 1976).

During chronic hypoxia acclimatization appears i.e. a progressive increase in ventilation in response to a given level of hypoxia is observed (Bisgard 1994, 1995, 2000). During the initial period of 30 min of hypoxia, there was an increase in ventilation that reduced $PaCO_2$ by 1-5 mmHg and at 8 h, hyperventilation increased bringing $PaCO_2$ to 8-10 mmHg. It was also observed that one CB is sufficient to elicit acclimatization and that the superfusion of intact CB with isocapnic hypoxic blood produces acclimatization, suggesting that CB hypoxia is the only factor required to elicit acclimatization in systemic hypoxia. When CSN discharges are recorded, they progressively increase during 5-6 h of sustained hypoxia (Bisgard 1994, 1995, 2000) with the acclimatization phenomena also being observed. The function and adaptation of the CB in response to chronic sustained hypoxia will be detailed further in Section 1.5.2.

The response of CB to anemic and ischemic (stagnant) hypoxia is physiologically unimportant. Very intense anemias and hypotension, which are barely compatible with life, are needed to elicit an increase in the CSN chemosensory activity or ventilation as long as PaO_2 is normal. Nevertheless, the aortic chemoreceptors respond to anemic and stagnant hypoxias of moderate intensities (Figure 6B and C), but the effects of aortic chemoreceptor activation on ventilation are negligible. In fact, direct electric stimulation of the aortic nerve did not modify phrenic nerve output in anesthetized dogs (Hopp et

al., 1991). The reasons for the distinct behaviour of CB and aortic bodies is not known, but it has been suggested as having a basis in the higher ratio of parenchyma volume to blood vessels in the aortic bodies in comparison with the CB, which implies a much lower blood flow to the aortic bodies than to the CB (Kumer and Addicks, 1986). This implies that aortic bodies detect rates of O_2 delivery, this being primarily responsible for the circulatory reflex responses, inducing several effects in order to prevent tissular hypoxia, like tachycardia, increased heart rate and increased arterial pressure (for a review see Gonzalez et al., 1994).

1.4. Function and control of the carotid body in hypercapnia and acidosis

The CB plays a homeostatic role in the responses to high PCO_2 and low pH in conjunction with central chemorecceptors allowing the pumping out of CO_2 at a rate matching its production, and allowing the maintenance of pH. Exercise is the only physiological situation associated with an increased CO_2 production and the CB plays an important role in exercise hyperventilation and in maintaining $PaCO_2$. In exercise the blood gases and pH are kept normal, but several pathological situations exist, like respiratory and metabolic acidosis, in which $PaCO_2$ and/or $[H^+]i$ increase, producing hyperventilation. Therefore, hypercapnia and acidosis are other natural stimuli for the CB.

It has been postulated that CB do not only sense changes in PCO_2 but in the $[H^+]i$. Thus, inhibition of carbonic anhydrase, the enzyme, present in chemoreceptor cells, that accelerates the hydration and dehydration of CO_2, thereby accelerating the changes in the $[H^+]i$ in response to PCO_2 alterations (Rigual et al., 1985), abolishes the dynamic increase in CSN chemosensory activity produced by sudden hypercapnia *in vitro* (Rigual et al., 1991; Buckler and Vaughan Jones, 1993) and *in vivo* (Nye et al., 1981, 1983), without modifying the steady response to CO_2 change. On the other hand, it is known that the release of CA evoked by weak acids in CB is approximately 80% dependent on extracellular Ca^{2+} (Rigual et al., 1991; Obeso et al., 1992) and Na^+ dependent, and inhibitable by blockers of proton extruding exchangers, supporting the sensing of $[H^+]i$ by chemoreceptor cells and not only the increase in PCO_2. Electrophysiological studies in rabbit and rat chemoreceptor

cells have shown that acidic stimuli are capable of depolarizing chemoreceptor cells and generate action potentials (Buckler and Vaughan Jones, 1993; 1994; Rocher et al., 2005) and found that hypoxia sensitive K^+ channels are also sensitive to acidosis (Peers and Green, 1991; Buckler et al., 2000). These findings lead to the conclusion that the transduction cascade in response to acidic stimulus-hypercapnia is as complex as the hypoxic transduction cascade.

At the level of CSN, it is known that discharges in CSN increase almost linearly with increasing $PaCO_2$ (or low pH) (Ponte and Purves, 1974; Rigual et al., 1991) but only up to certain levels, since at very high $PaCO_2$ (>100 mmHg) or very low pH (<6.9) the discharges tend to plateau. As Figure 8 shows, the slope of the lines relating $PaCO_2$ and CSN discharge increases as PaO_2 decreases indicating a positive interaction between both stimuli that is expressed in a multiplicative manner (Fitzgerald and Parks, 1971; Lahiri and Delaney, 1975a).

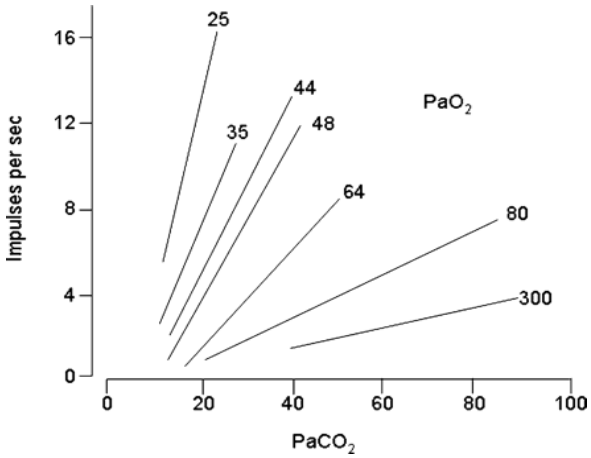


Figure 8 Relationship between afferent chemosensory discharge and CO_2 levels, recorded in carotid sinus nerve preparation. CO_2 levels were varied at different PO_2 levels. (Lahiri and Delaney, 1975a)

The relation between ventilation and $PaCO_2$ is similar to that encountered for the CSN discharge. However, in this situation, central chemoreceptors also contribute to the ventilatory responses. The CB has a lower threshold for PCO_2/acidic stimulus than central chemoreceptors, and so the hyperventilation observed in response to mild respiratory acidosis is originated mostly at the CB, but at more intense respiratory acidosis the CB only contributes 30-40% of the hyperventilatory response (see Gonzalez et al.,

1994). In response to a metabolic acidosis the contribution of CB is greater, especially in the initial periods since H^+ crosses the blood brain barrier slowly and has difficultly in reaching central chemoreceptors and exerting its effects (Irsigler et al., 1980). The hyperventilation trigered by CB decreases $PaCO_2$ and forces CO_2 to diffuse from the cerebrospinal fluid to blood. After several days of acidosis the pH of cerebrospinal fluid equilibrates with the blood stimulating both central and peripheral chemoreceptors (Cherniak and Altose, 1987).

1.5. Neurotransmission in the carotid body: role of adenosine, dopamine, ACh and ATP

Neurotransmission is the step by which chemoreceptor cells communicate with the sensory nerve endings, generating increases or decreases in CSN frequency activity. Neurotransmission also compromise the effect of neurotransmitters on the autoreceptors present in chemoreceptor cells.

CB contains several neurotransmitters, such as ACh, DA, ATP, 5-HT, substance P, endothelin-1, encephalin and nitric oxide (NO) (Buttigieg and Nurse, 2004; Conde and Monteiro, 2006; Gonzalez et al., 1994; Zhang et al., 2000).

In this section we are going to focus only on the neurotransmitters involved in the present work: adenosine, ATP, dopamine and acetylcholine; and on the effects of these mediators on chemotransduction and ventilation.

1.5.1. Adenosine

Adenosine is a product of ATP metabolism, which can be recycled to resynthethise ATP itself. Adenosine is not stored or released as a classical neurotransmitter and is an ubiquitous substance, being released by almost all cells and involved in key pathways such as purinergic nucleic acid base synthesis, amino acid metabolism and modulation of cellular metabolic status (Stone, 1985). Adenosine modulates the activity of several systems at presynaptic level (inhibiting or facilitating neurotransmitter release), at post-synaptic or at non-synaptic level (e.g. modulating blood flow or the metabolism of sustentacular cells).

1.5.1.1. Metabolic pathways of adenosine formation and release

Extracellular adenosine comes from the extracellular production through ATP catabolism via 5'-ectonucleotidases, as well as by its intracellular production and release by nucleoside transport systems. Intracellular adenosine production is mediated by an intracellular 5'-nucleotidase that dephosphorylates 5'-adenosine monophosphate (AMP) (Schubert et al., 1979; Zimmermann et al., 1998) or by the hydrolysis of S-adenosylhomocysteine (Figure 9). Beyond ATP catabolism, another source of extracellular adenosine is cAMP that can be released by secretory cells and converted by extracellular phosphodiesterases in AMP and then by a 5'-ectonucleotidase into adenosine (for a review see Fredholm et al., 2001).

Two families constitute the nucleotide transport system, which is bi-directional: one Na^+ independent and the other dependent on the same ion (Griffith and Jarvis, 1996). The Na^+ dependent nucleoside transport system is concentrative, concentrating nucleosides against a concentration gradient, being composed of five subfamilies in accordance with the selectivity of the substrate. The Na^+ independent family of nucleoside transporters (equilibrative nucleoside transport system) is formed of two different groups (*es* and *ei*), differentiated by their sensitivity to nitrobenzylthioinosine (NBTI) (Griffith and Jarvis, 1996; Cass et al., 1998, Podgorska et al., 2005). The *es* transport system is inhibited by nanomolar concentrations of NBTI and the *ei* requires micromolar concentrations to be inhibited (Griffith and Jarvis, 1996; Cass et al., 1998; Podgorska et al., 2005). Equilibrative nucleoside transport (ENT) consists of 4 different types of transporters, ENT1, ENT2, ENT3 and ENT4. ENT1 and ENT3 are susceptible to NBTI, whereas ENT2 is insensitive. ENT4 has been identified recently and its transport characteristics have not yet been fully characterized (Podgorska et al., 2005).

As a whole, these enzymatic transport pathways allow the conversion of minor changes in intracellular ATP into disproportionally large changes in extracellular adenosine concentrations.

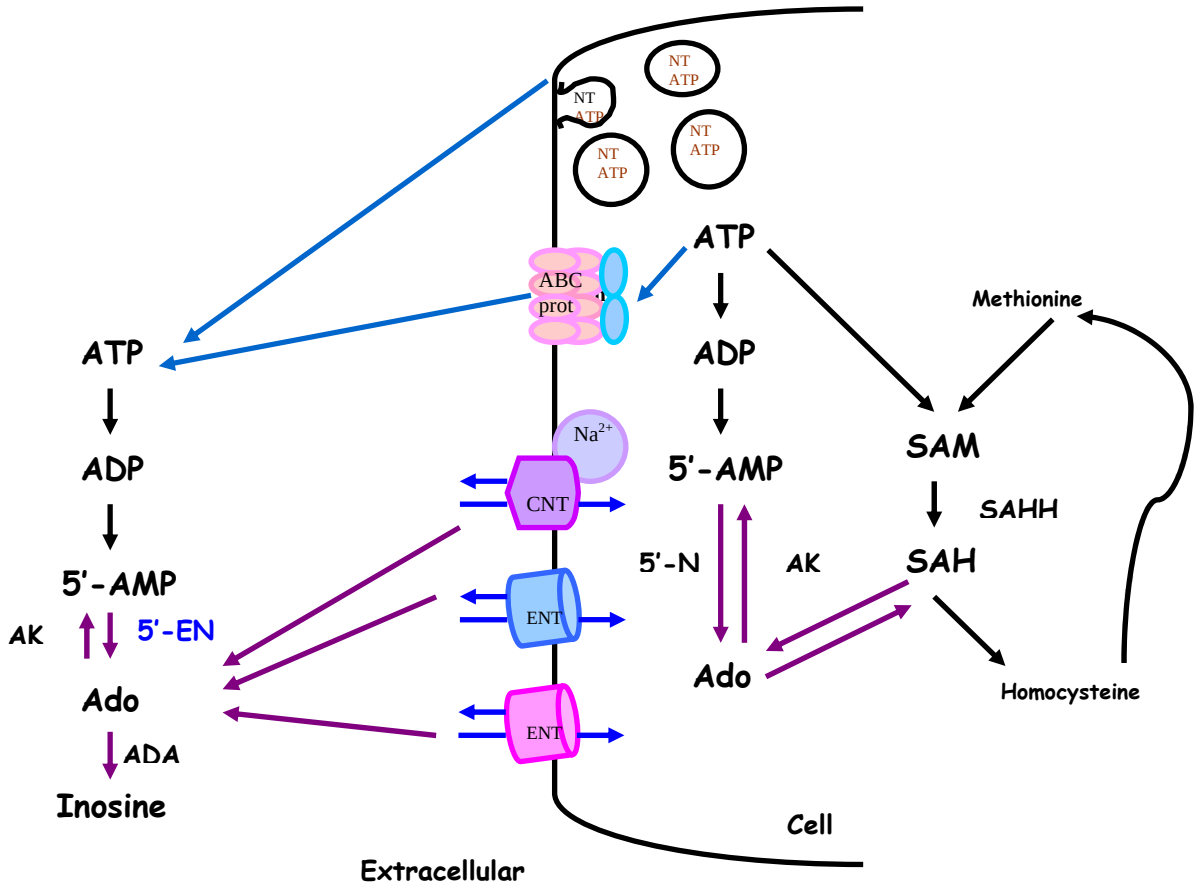


Figure 9 Extra- and intracellular adenosine metabolism and the nucleotide transporters that contribute to its release and uptake. 5'- EN: 5'-ectonucleotidase; 5' – N: 5'-nucleotidase; ABC protein: ATP transporter; ADA: adenosine deaminase; AK: adenosine kinase; CNT: concentrative nucleotide transport system; ENT1: type 1 equilibrative nucleotide transport system; ENT2: type 2 equilibrative nucleotide transport system; SAH: S-adenosylhomocysteine; SAHH: S-adenosylhomocysteine hydrolase; SAM: S-adenosylmethionine; NT: neurotransmitter.

1.5.1.2. Adenosine receptors

Adenosine exerts is action through the physiological activation of high–affinity adenosine receptors, A_1 and A_{2A} and lower-affinity A_{2B} and A_3 (for a review see e.g. Sebastiao and Ribeiro, 2000). In addition to the direct effect on the cells, adenosine receptor activation could indirectly influence the actions of other neurotransmitters and modulators. A_{2A} and A_{2B} receptors preferentially activate G protein from the G_s family, leading to an increase in cAMP, and A_1 and A_3 receptors that activate $G_{i/o}$ proteins, thus decreasing cAMP levels. In addition there is some evidence that the adenosine receptors may signal via other G proteins (Table 1). It has recently been described that one adenosine

receptor, the A_{2A} receptor, may be coupled to different G proteins in different areas of the body (Kull et al. 2000). Nevertheless, in the periphery, the receptor is thought to be coupled to the Gs. The activation of the G proteins leads to effects on enzymes and ion channels (see Table 1). A_1 receptors mediate inhibition of adenylyl cyclase, activation of several types of K^+ channels, inactivation of N, P and Q-type Ca^{2+} channels, activation of phospholipase C, etc. The same seems to happen with A_3 receptors. Both A_{2A} and A_{2B} receptors stimulate the formation of cyclic AMP, but other actions, including mobilization of intracellular calcium, have also been described. The distinct adenosine receptors, A_1, A_{2A}, A_{2B} and A_3 are activated by different endogenous adenosine concentrations (Fredholm et al., 1994). The disposable endogenous adenosine to activate these receptors and the quantity of adenosine receptors at the site of action help to control the distinct physiological responses to this nucleotide.

Table 1 G protein coupling of the four adenosine receptor subtypes (for a review see Fredholm et al., 2001).

Adenosine receptor subtype	G protein	Effects of G protein coupling	Cellular system
A_1	$G_{i1/2/3}$	↓cAMP	General, CHO cells
		↑IP_3/DAG (PLC)	
		↑Arachidonate (PLA_2)	
		↑choline (PLD)	DDT_1MF-2
	G_o	-	
A_{2A}	G_s	↑cAMP	General
	G_{olf}	↑cAMP	Striatum, CHO cells
	$G_{15/16}$	↑IP_3	COS-7
A_{2B}	G_s	↑cAMP	General
	$G_{q/11}$	↑IP_3/DAG (PLC)	HMC-1, HEK293
A_3	$G_{i2,3}$	↓cAMP	General, CHO cells
	$G_{q/11}$	↑IP_3/DAG (PLC)	CHO cells

Since adenosine plays modulatory roles in a variety of tissues and physiological circumstances it is expected that this nucleoside could act in concert with other several messengers and so, interactions between receptors occur. It is known that adenosine acts synergistically with other nucleotides like ATP and uridine 5'-diphosphate (UDP), and histamine or bradykinin (Fredholm et al., 2001). Interactions between A_1 and D_1 and NMDA receptors are also

described in the brain. The best-known interaction with adenosine receptors is the interaction between A_{2A} and D_2 receptors that is present in the basal ganglia, this being a promising therapeutic target for the treatment of Parkinson's disease. A more detailed review concerning the interactions between A_{2A} and D_2 receptors is in section 1.5.2.2..

1.5.1.3. Physiological role of adenosine

Adenosine regulates many physiological processes, particularly in excitable tissues such as heart and brain. Many of the actions of adenosine either reduce the activity of excitable tissues (e.g. by slowing the heart rate) or increase the delivery of metabolic substrates (e.g. by inducing vasodilatation) and, thus, help to couple the rate of energy expenditure to the energy supply (refrain cell metabolism). Most often, this adenosine-mediated inhibition of cell metabolism is mimicked by A_1 receptor agonists, which leads to the idea that adenosine-induced inhibition of cell metabolism is mediated by A_1 adenosine receptors. The adenosine-mediated excitation is thought to be due to an action on A_2 adenosine receptors. These roles of adenosine do not explain all its actions, as it also has several roles as an intracellular messenger.

Adenosine has diverse roles in normal physiology, such as promoting and/or maintaining sleep (Portas et al., 1997; Lin et al., 1997), regulating the general state of arousal (Rainnie et al., 1994) as well as local neuronal excitability (Fredholm et al., 2005), and coupling cerebral blood flow to energy demand (Phillis, 1989). Selective adenosine receptor antagonists have been used frequently in the past to provide evidence concerning these proposed roles for adenosine. The recent development of knockout mice for the A_{2A} receptor, A_3 receptor and A_1 receptor has provided additional tools to characterize the functions of these receptors over the last few years (for a review see Fredholm et al., 2005). In pathological conditions, adenosine is a neuroprotective agent against hypoxic or ischemic events (for a review see Latini and Pedata, 2001) and seizure-induced neuronal injury (Dunwiddie, 1999). The fact that adenosine produced intra- or extracellularlly can be released from several tissues and preparations, like rat striatal slices, hippocampal slices and glial and PC12 cells in culture, under ischemic and

hypoxic conditions (Fowler, 1993; Lloyd et al.; 1993; Jin and Fredholm, 1997; Kobayashi et al., 2000; Koos et al., 1997; Medhji et al., 1989), leads us to propose that CB can also release adenosine in response to hypoxia, this mediator contributing to the mechanisms of chemoreception during hypoxic signalling.

1.5.1.4. Adenosine in chemoreception in the carotid body

Adenosine is capable of stimulating the CSN chemosensory activity, as was first described in 1981 by McQueen and Ribeiro. After that pioneer study, much experimental evidence has been obtained supporting the idea of an important role for adenosine in chemoreception. For example, adenosine applied exogenously to cat CB preparations increases CSN discharges both *in vivo* (McQueen and Ribeiro, 1983) and *in vitro* (Runold et al., 1990). In the rat, intracarotid injections of adenosine activate ventilation, an effect suppressed by CSN section and mediated by A_2 receptors (Monteiro and Ribeiro, 1987); this *in vivo* excitatory effect of exogenous adenosine is mimicked by drugs that increase levels of endogenous adenosine, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and dipyridamole, inhibitors of adenosine deamination and uptake, respectively (Monteiro and Ribeiro, 1989). The importance of these effects of adenosine on chemoreception, described in rats and cats, was strongly reinforced by the results obtained in humans. Intravenous infusion of adenosine in healthy volunteers causes hyperventilation, dyspnoea and chest discomfort (Watt and Routledge, 1985, Uematsu et al., 2000). This effect of adenosine in man is attributed to the activation of CB chemoreceptors because: 1) adenosine, when applied intravascularly does not cross the blood brain barrier (Berne et al., 1974); 2) its effect on ventilation is proportionally higher the closer to the CB the bolus of adenosine is administered (Watt et al., 1987), and; 3) adenosine and its antagonists modify the hyperventilatory responses to hypoxia that is completely mediated by the CB (Maxwell et al., 1986; Maxwell et al.,1987).

The chemoexcitatory effect of adenosine appears to be mediated, at least in part, via modifications in cyclic AMP (cAMP) levels. Thus, adenosine increases cAMP content in the rat CB (Monteiro et al., 1996), and the increase

in cAMP levels (e.g. by the administration of cAMP analogs resistant to enzymatic degradation) potentiates the response to hypoxia (Perez Garcia et al., 1991). It is also known that dipyridamole, an inhibitor of adenosine uptake that increases the effective concentration of adenosine in the extracellular milieu, potentiates the augmentation of cAMP produced by hypoxia; this hypoxic response is blocked nearly completely by A_2 antagonists (Chen at al., 1997).

Immunocytochemical studies have demonstrated the expression of A_{2A} receptors and its co-localization with tyrosine hydroxylase (TH) in rat CB chemoreceptor cells (Kobayashi et al., 2000a; Gauda et al., 2000), and RT-PCR techniques have detected the presence of mRNA for A_{2A} and A_{2B} receptors in rat CB homogenates (Kobayashi et al., 2000a). In addition, Rocher and co-workers demonstrated the presence of A_1 receptors in the rabbit CB chemoreceptor cells by showing that A_1 agonists and antagonists are capable of modulating Ca^{2+} currents in these cells (Rocher et al., 1999). However, A_1 receptors appear to be absent in rat CB (Kobayashi et al., 2000a; Gauda et al., 2000).

Recently, Fitzgerald and co-workers (Fitzgerald et al., 2004) have suggested that adenosine effects on ventilation are due to its action as a modulator of the release of the classical neurotransmitters, enhancing the release of what is accepted as a stimulatory neurotransmitter in the cat carotid body, ACh, and depressing the hypoxia-induced release of a transmitter which is accepted to be inhibitory, DA. It is known that the release of classical neurotransmitters is extracellular Ca^{2+} dependent, and it has been demonstrated by Kobayashi et al. (2000b) that adenosine did not alter $[Ca^{2+}]_i$ in cells exposed to normoxia. However, a recent study in rat chemoreceptor cells has shown that adenosine via A_{2A} receptors triggers a small increase in intracellular Ca^{2+} levels (Xu et al., 2006), but the increase in the intracellular Ca^{2+} observed by these authors seems to be insufficient to reach the threshold to evoke a release of neurotransmitters (Vicario et al., 2000b), so it is hard to believe that adenosine effects on the CB can only be explained through the modulation of the release of both ACh and DA. In fact, the excitatory role of adenosine in chemoreception in the CB would be reinforced if this mediator were also involved in the specificity of the response of CB chemoreceptors to acute and/or chronic hypoxia.

In this work we have tried to establish a role for adenosine in CB chemoreceptor function in response to acute hypoxia, investigating: whether hypoxia specifically releases adenosine from chemoreceptor cells; the effect of adenosine and its antagonist caffeine on the release of CA and on the CB output in basal and hypoxic conditions, thereby studying the identity of the adenosine receptors involved in adenosine and caffeine effects on CB chemoreceptors; and, finally, if adenosine release is modulated by other neurotransmitters. These points will be treated in Chapter 1, Chapter 2 and Chapter 3 of the present work. In order to establish a role for this neurotransmitter in CB chemoreception in chronic hypoxia we have permanently blocked adenosine receptors with a chronic caffeine treatment and studied CB function at several levels (see Section 1.6.2., Chapter 5 (Section 7)).

1.5.2. Dopamine

Catecholamines (CA), DA, NE and epinephrine (E) are classical neurotransmitters of the central and peripheral nervous system. NE is a neurotransmitter in the brain as well as in sympathetic postganglionic neurons. DA is an NE precursor, being one of the main transmitters in the central nervous system, but it has also been reported as playing an important role in the peripheral system.

1.5.2.1. CA synthesis and metabolism

The enzymatic synthesis of CA is well characterized; it is known that TH is the rating enzyme for CA biosynthesis. TH is present in all cells that synthesize CA, being a mixed function oxidase that uses molecular oxygen and tyrosine as substrates and tetrahydrobiopterin as a co-factor to produce 3,4,dihydroxy-L-phenylalanine (L-DOPA), this step being the rate limiting step in dopamine biosynthesis. L-DOPA is converted to DA by the amino acid decarboxylase (also known as aromatic amino acid decarboxylase, AADC) (Figure 10), this being the last step in the biosynthesis pathway in dopaminergic neurons. In noradrenergic and adrenergic neurons, dopamine-ß-hydroxylase (DBH) is the next enzyme in the pathway.

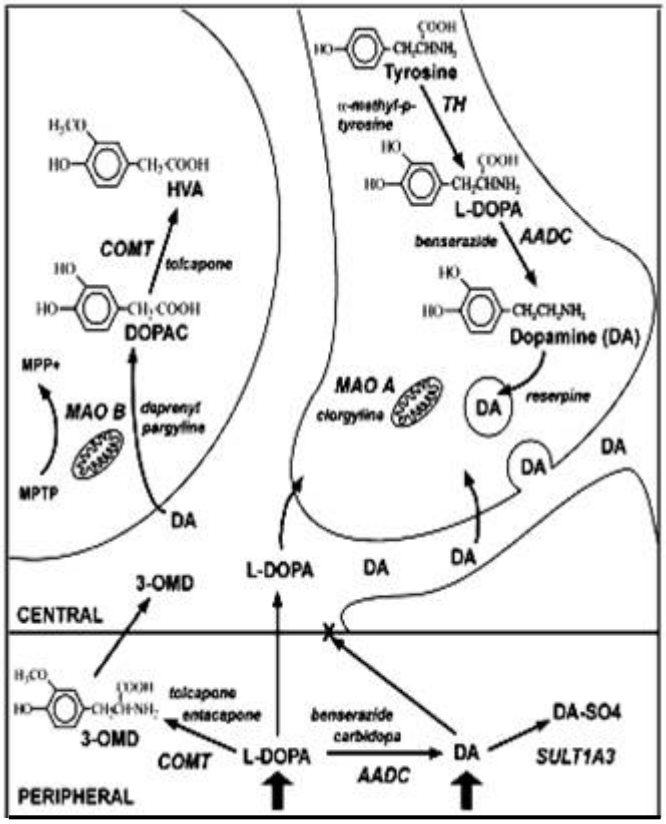


Figure 10 Synthesis and metabolism of dopamine (DA) in humans. Tyrosine hydroxylase catalyzes L-DOPA formation from tyrosine in dopaminergic presynaptic neurons. L-DOPA is converted into dopamine by amino acid decarboxylase (AADC) and incorporated into vesicles through the vesicular monoamine transporter (VMAT). After DA release in the synapse, DA returns to presynaptic component by reuptake by the DA transporter (DAT) (Oak and Van Tol, 2004).

In the same manner as TH, DBH is a mixed function oxidase that uses molecular O_2 to form a hydroxyl group that is added to ß carbon in the lateral chain of DA. Ascorbate is reduced to dehydroascorbate in the reaction, giving an electron to the overall reaction. DBH is concentrated in the vesicles that contain CA, most of the time being attached to the inner vesicular membrane. DBH is released with CA by neurons and by cells of adrenal medulla.

In cells that synthesize E, the final step of the biosynthesis pathway is catalyzed by the enzyme phenylethanolamine-N-Methyltransferase. This enzyme is present in a small group of brain stem neurons that use epinephrine as a neurotransmitter and in cells of adrenal medulla that use epinephrine as a primary neurohormone. This enzyme transfers a methyl functional group of S-adenosyl-L-methionine to the nitrogen of NE, producing a secondary amine (for a review see e.g. Kuhar et al., 1999).

1.5.2.2. Dopamine receptors

Dopamine exerts its physiological actions through the DA receptors that are divided into two families according to their action on adenylyl cyclase (AC) and thereby on the levels of cAMP. Functionally, the D_1-like receptors (D_1 and D_5) are coupled to Gαs proteins and can stimulate adenylyl cyclase. The D_2-like receptors (D_2, D_3 and D_4) are coupled to $G_{i/o}$ proteins and consequently inhibit AC, producing a decrease in cAMP levels. While D_1 receptors act almost exclusively through the $G_{\alpha s}$–mediated activation of AC, the D_1-like receptors exert their effect through other pathways like calcium channels, potassium channels, arachidonic acid, sodium-hydrogen exchanger, etc..

Through a mechanism of alternative splicing, the D_2 receptor gene encodes two molecularly distinct isoforms, D_{2S} (short) and D_{2L} (large). These isoforms could interact differently with G proteins originating different *in vivo* effects (Picetti et al., 1997; Usiello et al., 2000). The D_{2S} receptors would be present mostly presynaptically and would counteract D_1-mediated effects; D_{2L} receptors would be post-synaptic (Usiello et al., 2000).

Direct interactions between dopamine receptors and other receptors have also been described. For example, interactions of D_1 receptors with AMPA receptors modulating synaptic plasticity in the hippocampus (Gao et al. 2006); and between D_2 receptors and A_{2A} receptors in the striatum and hippocampus, these interactions giving the possibility of therapeutic targets for disorders like Parkinson's disease, Huntington's disease and schizophrenia (for a review see Sebastiao and Ribeiro, 2000; Fredholm et al., 2005).

These interactions between A_{2A} and D_2 receptors exist in several central dopaminergic systems. For example, in the rat striatum it was observed that A_{2A} adenosine receptor activation diminished the affinity of D_2 receptors for its agonists (Ferré et al., 1991). It was also observed that A_{2A} receptor agonists diminished the coupling of D_2 receptors to G proteins (Ferré et al., 1993) and that activation of D_2 receptors antagonized A_{2A} signalling (Fuxe et al., 2001). In addition, an augmentation in the interaction A_{2A}-D_2 in the rat striatum after depletion of striatum dopamine or chronic treatment with D_2 antagonists has been demonstrated (Ferré and Fuxe, 1992; Ferré et al., 1994). These data suggest there is a molecular basis for these A_{2A}-D_2 interactions at the cell

membrane level. The evidence of this interaction between A_{2A}-D_2 receptors is supported by *in situ* hybridization studies and confocal microscopy that suggest that these 2 types of receptors are collocated in the cell membranes and that chronic exposure to D_2 and A_{2A} agonists lead to co-aggregation, co-internalization and co-desensitization of both receptors (Hillon et al., 2002). Both A_{2A} and D_2 receptors are coupled to adenylyl cyclase, and so the interaction between these two neurotransmitters can also be seen at second messenger levels and through other intracellular transducer signalling systems, like c-fos and jun-B (for a review see Xu et al., 2005).

1.5.2.3. Dopamine effects on chemoreception and ventilation

The detection of high levels of dopamine within the CB of all mammalian species led several research groups to study the effects of this amine on chemoreflexes and chemosensory activity. Concerning the effects of dopamine and its agonists and antagonists on ventilation, we must distinguish peripheral effects from central effects. Initial studies on the effect of DA on ventilation have suggested an excitatory effect of this amine on ventilation in dogs (Black et al., 1972; Jacobs and Comroe, 1968). However, later publications indicate that intravenous or intracarotid injections or infusions of DA mostly depress ventilation in cats, dogs, rats, goats and newborn lambs (for a review see Zapata, 1997), with the effect disappearing after CSN section. Thus, ventilatory depression was attributed to the reflex effects of DA on chemosensory impulses. It has also been described that the application of domperidone, a D_2 dopamine receptor antagonist, that does not cross the blood brain barrier, produces, via an action on CB pre- and post-synaptic receptors (Dinger et al., 1981b; Mir et al., 1984), an increase in ventilation (Bee and Pallot, 1995) and in the CSN chemosensory activity (Fidone et al., 1997; Hsiao et al., 1989; Iturriaga et al., 1994; Tatsumi et al., 1995b), these actions being in accordance with an inhibitory effect of DA at the CB. Nevertheless, it is known that low doses of DA depress CSN activity but high doses have an excitatory effect (Zapata 1975, for a review see Zapata, 1997). The physiological importance and the reasons for these differences of effect have been discussed in terms of presence and

function of the distinct types of receptors present in the CB and in terms of signal transduction pathways associated with these receptors.

Contrary to what happens with a peripheral administration, increases in ventilation were observed with intracerebroventricular application of D_2 receptor agonists in the rat (Hedner et al., 1982). Intracerebroventricular administration of domperidone and haloperidol, other antagonists of D_2 receptors, which cross blood brain barrier, inhibit normoxic ventilation (Hedner et al., 1982).

It is described that CA synthesis, particularly of DA, increases in response to acute (Fidone et al., 1982) and chronic hypoxia (Pequinot et al., 1987) suggesting an increase of its utilization in response to the CB physiological stimuli. As previously described in section 1.1, it is known that all the stimuli that activate CB (hypoxia, hypercapnia, cyanide, high extracellular K^+) release DA in all mammalian species, the release being proportional to stimulus intensity, to the increase in CSN activity and dependent on extracellular Ca^{2+} (Figure 11) (Fidone et al., 1982; Obeso et al., 1985, 1986, 1992, 1999; Rigual et al., 1986, 1991, 2002; Rocher et al., 1991; Vicario et al., 2000a; Sanz-Alfayate, 2001).

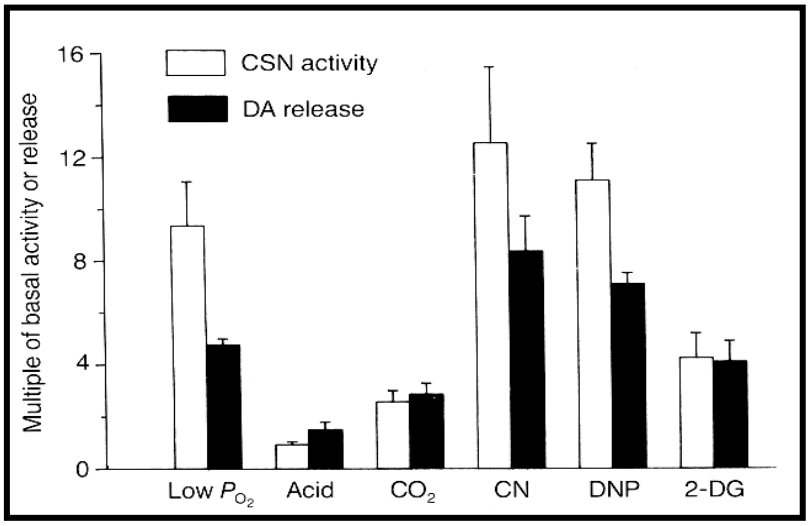


Figure 11 Effects of different stimuli on the release of ^{3}H-DA and CSN activity in an *in vitro* preparation of cat carotid bodies. (Gonzalez et al., 1992).

The DA released from CB acts on DA autoreceptors present in the organ. Autoradiographic, immunohistochemical, in situ-hibridization and RT-PCR studies have demonstrated the presence of D_2 dopamine receptors on sensorial nerve endings and in type I cells of distinct species (Bairam et al., 1997; Dinger

et al., 1981b; Gauda et al., 1994, 2000; Mir et al., 1984). The two isoforms of D_2 dopamine receptors are expressed in chemoreceptor cells, the D_{2L} isoform being predominant (Bairam and Khandjian, 1997). Knowing that D_{2S} are located presynaptically and D_{2L} are almost exclusively post-synaptic, it is postulated that DA released by chemoreceptor cells is controlled by a concentration-dependent negative feedback mechanism mediated by D_{2S} autoreceptors (Bairam et al., 2000).

Concerning D_1 dopamine receptors, their presence in the CB has been described (Almaraz et al., 1991; Bairam et al., 1998). Nevertheless their levels of expression are much lower than for D_2 receptors, and they also present a lower affinity for dopamine (Bairam et al., 1998). The effects of dopamine on chemoreception are reinforced by the observation that the activation of both D_1 and D_2 receptors modulates cAMP levels in chemoreceptor cells: D_1 agonists increase cAMP (Almaraz et al., 1991) and D_2 agonists decrease it (Batuca et al., 2003).

Regarding the interactions between A_{2A} and D_2 receptors in the CB, Ribeiro and McQueen (1983) in their pioneer study on the effects of adenosine, had observed some kind of interaction between the dopaminergic and adenosinergic systems, since adenosine potentiates the inhibitory effects of dopamine on CSN discharges in the cat. Strong evidence for the existence of these interactions has been observed more recently *in vivo* in the rat CB, since adenosine heightens the inhibitory effect of dopamine in ventilation mediated by CB chemoreceptors, the adenosine A_2 antagonist DPSPX decreases this inhibitory dopamine effect, and domperidone, which is a D_2 antagonist, reduces the excitatory effect of adenosine on ventilation (Monteiro and Ribeiro, 2000). It was also observed by these authors that in ischemic conditions, where endogenous adenosine levels are increased, the inhibitory effect of D_2 agonists can be attenuated by the blockage of A_2 receptors, converting the dopaminergic effect into an increase in ventilation (Monteiro and Ribeiro, 2000).

As described in section 1.5.1.4., it was suggested by Fitzgerald et al. (2004) that the effects of adenosine in ventilation are due to the modulation of the release of several neurotransmitters, like ACh and DA. Following this line of thought we decided to investigate the effects of adenosine on the release of CA and, since interactions between A_2 and D_2 receptors are described in the CB, to

investigate whether they could modulate this release. This point will be addressed in Chapter 4 of the thesis.

1.5.3. ATP

Adenosine triphosphate (ATP) has a fundamental intracellular role as the universal source of energy for all living cells. The demonstration of its release into the extracellular space and the identification and localisation of specific receptors on target cells have been essential in establishing its extracellular physiological roles. It is now accepted that ATP is a genuine neurotransmitter both in the central and peripheral nervous systems. As a classical neurotransmitter, the release of ATP by nerve terminals is produced by exocytosis. Recent evidence, however, suggests that in some non-neuronal cells, ATP could also be released by a carrier-mediated transport and would involve ATP-binding cassette proteins (ABC), an ubiquitous family of transport ATPases (for a review see Bodin and Burnstock, 2001).

ATP exerts its physiological actions through the activation of its receptors, that are divided in two families: P2X if the receptor is ionotropic and P2Y if is metabotropic. This nomenclature has been widely adopted and currently there are seven P2X ($P2X_1$-$P2X_7$) subtypes described and eight P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) subtypes (King and Burnstock, 2002; North, 2002).

1.5.3.1. Role of ATP in chemoreception in the carotid body

Recently ATP has been assigned an important role in chemotransduction in the CB, as an excitatory neurotransmitter. The first study on the effects of ATP on CSN activity, by McQueen and Ribeiro (1983), observed that this nucleotide increased CSN activity in a dose-dependent manner. However, this effect was attributed to adenosine formed by the catabolism of ATP, since the non-hydrolysable agonist of ATP, α,ß-MethyleneATP (10 - 100 µg i.c.), did not produce the same effect and in fact had diminished the CSN activity. Spergel and Lahiri (1993) have shown that ATP and 5'-γ-thio-triphosphate (ATP-γ-S) induce increases in cat CSN chemosensory activity with the response being

very transient. However, the possibility cannot be excluded that these compounds can suffer hydrolysis to form adenosine. In 1998, in a study designed to investigate whether the activation of P2X receptors associated with vagal afferent could induce a Bezold-Jarisch reflex (triad of apnoea, bradycardia and hypotension) in the rat, it was observed that α,ß-MethyleneATP, a non-hydrolysable agonist which is relatively selective for particular subtypes of P2X receptor, and ATP excited CB, causing reflex hyperventilation (McQueen et al., 1998). More recently, Zhang and co-workers (Zhang et al., 2000) have demonstrated in co-cultures of chemoreceptor cells and "juxtaposed" petrosal neurons that the co-application of hexamethonium and suramin, blockers of nicotinic and P2X receptors, respectively, completely abolished spontaneous post synaptic activity in response to hypoxia, suggesting a possible co-transmission of ATP and ACh in chemoreceptor cells. This theory of ATP acting as co-transmitter was supported by the presence of $P2X_2$ and $P2X_3$ protein in many petrosal somas and CB afferent terminals in situ (Prasad et al., 2001). The idea that ATP has an excitatory effect on chemoreception was supported by the finding that mice deficient in $P2X_2$ showed a markedly attenuated ventilatory response to hypoxia (Rong et al., 2003), and by the finding that ATP is released from rat CB in hypoxia (Buttigieg and Nurse, 2004, Conde and Monteiro, 2006), this release being proportional to hypoxia intensity (Conde and Monteiro, 2006).

Adenosine is both a catabolic product and a precursor of ATP. Therefore, it was important to discern whether the effects of hypoxia and ACh on the release of adenosine from CB observed in this work were due to a release of adenosine by itself or due to a release and consequent hydrolysis of ATP. These points are dealt with in Chapters 1 and 2.

Since ATP is nowadays considered one of the excitatory neurotransmitters that contribute to CB chemoreception in acute hypoxia (Zhang et al., 2000; Rong et al., 2003; He et al., 2005; Zapata, 2007) we have also investigated the effects of chronic hypoxia and chronic caffeine treatments in its release from CB. This point will be addressed in Chapter 5, Section 7 of this work.

1.5.4. Acetylcholine

Acetylcholine was the first neurotransmitter ever identified as being a chemical transmitter in both the central and parasympathetic nervous systems. Chemically, acetylcholine is an ester of acetic acid and choline - $CH_3COOCH_2CH_2N^+(CH_3)_3$. It is a very effective deliverer of sodium ions, which depolarise and stimulate muscle contractions and excite nerves. Acetylcholine (ACh) is synthesized from choline and acetyl-CoA through the action of choline acetyltransferase (ChAT) (Figure 12). When ACh is released it must be removed rapidly in order to allow re-polarisation to take place; this step is carried out by hydrolysis by the enzyme acetylcholinesterase (AChE). The acetylcholinesterase found at nerve endings is anchored to the plasma membrane through a glycolipid.

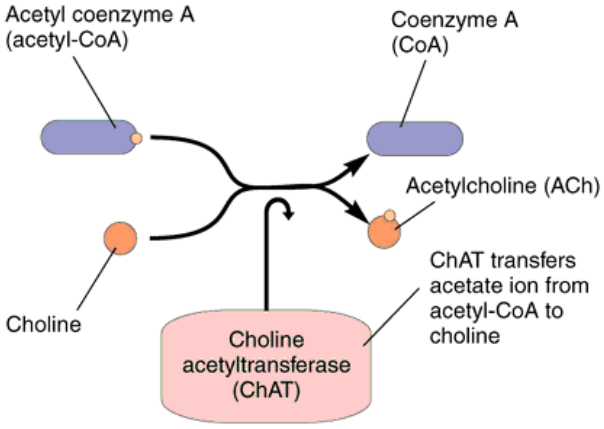


Figure 12 Biosynthesis pathway of acetylcholine formation.

ACh is involved in key functions in the body. It is the key mediator of electrical transmission creating the skeletal muscle tone at the neuromuscular junction; it is involved in several processes connected to cognitive functions, learning and memory, arousal, reward, motor control and analgesia in autonomic basal ganglia and cholinergic neurons through CNS; and in regulating the synaptic release of itself and other important neurotransmitters (for a review see e.g. Jensen et al., 2005).

1.5.4.1. Acetylcholine receptors

Two main classes of ACh receptors have been identified on the basis of their responsiveness to the toadstool alkaloid, muscarine, and to nicotine. They are, respectively, the muscarinic receptors and the nicotinic receptors. Both receptor classes are abundant in the human brain. Although the pharmacological differences between nicotinic and muscarinic receptors are quite substantial, they are both stimulated by acetylcholine, and by carbachol. Nicotinic receptors are pentameric subunit ion channels (Alexander at al., 2004; Karlin, 2002; Unwin, 2005) and muscarinic receptors are metabotropic receptors, and so, coupled to G-proteins (Alexander et al., 2004).

Muscarinic receptors are divided into five subtypes (M_1–M_5) that emerged from cloning experiments (Bonner *et al.*, 1987), with differential coupling to G-proteins (M_1, M_3, M_5 to the G_q/G_{11} family, and M_2, M_4 to the G_i/G_o family), and, to some degree, differential sensitivities to some antagonists (Caulfield and Birdsall, 1998; Alexander *et al.*, 2004). Muscarinic receptors are involved in a great variety of central functions, like basal ganglia motor activity, analgesia and hypothalamic function (temperature, feeding) (Brown, 2006).

Nicotinic receptors are ligand-gated ion channels mediating fast synaptic transmission of the neurotransmitter. The nicotinic receptors present in neuromuscular junctions are located mainly postsynaptically. However, neuronal nicotinic receptors are both presynaptic and postsynaptic, the presynaptic nicotinic receptors regulating the synaptic release of ACh and the release of other neurotransmitters such as DA, NE, 5-HT, glutamate and gamma aminobutyric acid (GABA) (for a review see e.g. Jensen et al., 2005).

Due to their modulatory role, nicotinic receptors have been proposed as potential therapeutic targets to treat pain, epilepsy, and a wide range of neurodegenerative and psychiatric disorders.

The nicotinic ACh receptors are ligand-gated ion channels composed of pentameric assemblies of subunits surrounding a central aqueous pore gating the flux of cations (Na^+, K^+ and Ca^{2+}). Seventeen nicotinic ACh receptors have been cloned. The subunits are divided into muscle type (α1, ß1, δ, γ, ε) and neuronal type (α2-α10 and ß2-ß4).

Table 2 Functional characteristics of selected nicotinic AChR ligands at neuronal nicotinic ACh receptors (Adapted from Jensen et al., 2005).

	α3ß2	α4ß2	α2ß4	α3ß4	α4ß4	α7
Agonists						
ACh	443, 126/150%, 26/100%	68, 100/172%	83	203, 163/100%	20	180
(*S*)-nicotine	132	5.5	21	8	5.0	113
(-)-cytisine	67	2.7	39	72	0.9	2.6
(()-epibatidine	0.443/163 %	0.043/117 %	0.010/115 %	0.15/267%	0.038/99 %	0.072/97%
DMPP	56	18	23	19	19	31
A-85380		0.7/185%		0.8/100%		8.9/100%
tebanicline		0.27/100%		0.20/116%		56/83%
Altinicline	3.2	0.32	3.2	10	2	>10
NFEP		0.013		0.32		25
GTS-21		>10		>10	>10	6
Antagonists						
α-bungarotoxin	>1	>1	>1	>1	>1	0.005
DH ß E	1.6, 87	0.11, 2.7	3.6, 5.1	14	0.01	20
d-tubocurarine	2.4, 0.23	3.2, 7.6	4.2, 2.3	2.2, 0.73	0.21, 0.86	3.1
MLA	>1	>1	>1	>1	>1	0.0017
A-186253		0.96		9.2		8.5
MG624		3.2				0.11
Allosteric inhibitors						
mecamylamine	0.28	0.78	3.1	1.4	0.56	12.3
Bupropion	≈1	≈10		1.8	14	≈50

EC50 and *R*max values are given for the agonists (in *μ*M and in % of the *R*max of ACh or (*S*)-nicotine) and *K*i or IC50 values are given for the antagonists (in *μ*M). The data have been obtained in electrophysiological recordings on nAChRs expressed in *Xenopus* oocytes or in mammalian cells by Ca^{2+} measurements using fluorescent dyes or in 86Rb+ efflux assays. The data are primarily from recombinant human nAChRs, although data from studies using recombinant rat or chick nAChRs or cell lines expressing native nAChRs have also been included. nd: not determinable

On the basis of their gene structures and protein sequences their subunits have been grouped into 4 subfamilies (I-IV), with subfamily 3 being

subdivided into three tribes: a) family I is composed of α9 and α10; b) family II is composed of α7 and α8; c) family III is subdivided into 1 (α2, α3, α4 and α6), 2 (ß2 and ß4) and 3 (ß3 and α5); d) family IV is composed of α1, ß1, δ, γ and ε (for a review see Jensen et al., 2005). Numerous compounds have been identified that act as either agonists or antagonists of cholinergic neurons (see Table 2).

The principal action of cholinergic agonists is the excitation or inhibition of autonomic effector cells that are innervated by postganglionic parasympathetic neurons and, as such, are referred to as parasympathomimetic agents. The cholinergic agonists include choline esters (such as ACh itself) as well as protein- or alkaloid-based compounds (Table 2). The responses of cholinergic neurons can also be enhanced by administration of AChE inhibitors (Zwart et al., 2000).

1.5.4.2. Effects of ACh on carotid body chemoreception

Among the hypotheses proposed to account for stimulus-generated neural activity from the carotid body, the Cholinergic Hypothesis was the first (Heymans, 1936).

Several authors have described the presence of ACh in the carotid body (Eyzaguirre et al., 1965; Fidone et al., 1976; Hellstrom, 1977) and demonstrated that section of CSN did not lower the ACh content of CB (Fidone et al., 1976), suggesting that ACh is present almost exclusively in the CB. Along the same line of observation, Hellstrom (1977) showed that the removal of SCG does not modify the CB ACh levels and Shirahata et al. (1996) observed that ACh is released from both cat and pig chemoreceptor cells in culture, although, in these cultures, the release from other cell types cannot be excluded. Taken together these results indicate that the ACh present in the CB would be in chemoreceptor cells or in type II cells. Immunocytochemical studies have demonstrated the presence of ChAT in cat and rabbit chemoreceptor cells (Wang et al., 1991). However, Almaraz's cat studies in 1983 did not observe ACh synthesis or ChAT activity. Regarding the presence of choline, a precursor of ACh, a large amount is described in CB preparations (Fidone et al., 1987;

Fitzgerald, 2000). More recently, Nurse and Zhang (1999) have described the expression of ChAT and AChE in the chemoreceptor cells, indicating that enzymatic machinery for the generation and inactivation of ACh is present in the CB chemoreceptor cells. Despite all these findings regarding the presence of the enzymes involved in the biosynthesis pathway of ACh, Gauda (Gauda, 2002; Gauda et al., 2004a) has reported that chemoreceptor cells are negative for ChAT and also to vesicular ACh transporter (VAChT), suggesting that ACh comes from nerve endings or other cell types instead of chemoreceptor cells. These controversial findings can be explained if chemoreceptor cells undergo phenotypical changes in co-cultures.

Hypoxia results in a modest release of ACh from cat CB (Fitzgerald at el., 1999). In rat and rabbit CB, hypoxia inhibits the basal release of ACh via activation of muscarinic and dopaminergic autoinhibitory receptors in the CB (Kim et al., 2004). These results are in agreement with earlier studies in the central and peripheral nervous system in which it was observed that hypoxia inhibits ACh release (Chleide and Ishikawa, 1990; Gibson and Peterson, 1982).

Concerning the presence of nicotinic ACh receptors at the CB, it is known that they are present in chemoreceptor cells (Dinger et al., 1981a; Dasso et al., 1997; Obeso et al., 1997a) and in nerve fibres (Shirahata et al., 1998). Immunohistochemical and RT-PCR techniques in the cat have shown the presence of α_3, α_4 and β_2 subunits in chemoreceptor cells (for a review see Higashi *et al.*, 2003) and α_7 subunits in nerve fibres surrounding the chemoreceptor cells (Shirahata et al., 1998). In mice, transcripts of six nicotinic ACh receptor subunits, α_3, α_4, α_5, α_7, β_2 and β_4, were detected in carotid body total RNA (Cohen et al., 2002). The physiological function of these receptors is not completely understood but they may be involved in the hyperpnoea caused by exogenous nicotine (Fernández et al., 2002). Until the nineties nicotinic receptors were not considered fundamental for the O_2 sensing mechanism at the CB because classical nicotinic blockers suppressed the excitatory actions of nicotinic agonists, but only reduced the activation produced by natural stimuli by a variable percentage (McQueen 1977; 1983). More recently, Nurse and co-workers (Zhang et al., 2000) showed that the application of nicotinic ACh antagonists, like mecamylamine or hexamethonium, in co-cultures of glomus cells and "juxtaposed" petrosal ganglions only partially inhibits the hypoxia

evoked excitatory postsynaptic responses. Nevertheless, it is agreed that nicotinic ACh receptors in glomus cells act as modulators, increasing $[Ca^{2+}]_i$ (Dasso et al., 1997) and inducing the release of neurotransmitters at the CB, such as dopamine (Obeso et al., 1997).

ACh was the first neurotransmitter proposed in CB synapsis. Nonetheless, the incomplete blockage of nicotinic antagonists in hypoxic CB response and the absence of expression of enzymes essential for the ACh biosynthesis pathway in the CB, leads us to question the Cholinergic Hypothesis (see Zapata, 2007). However, the excitatory effects of ACh on CB could involve the release of other excitatory neurotransmitters and the importance of adenosine/ATP in CB chemotransduction would be reinforced if it were also involved in the chemotransduction mechanism originated by substances like nicotine/ACh that apparently mimic the excitatory effect of hypoxia on CSN activity and/or ventilation. This point will be addressed in Chapter 2, Section 4 of the present work.

1.6. Effects of chronic hypoxia

Chronic hypoxia must be divided into intermittent and sustained hypoxia. Chronic intermittent hypoxia is characterized by transient episodes of hypoxia of small duration. People who reside at or near the sea level are more predisposed to recurrent episodes of hypoxia than to sustained hypoxia. Transient episodes of hypoxia can occur, for example, with air swallowed during meals that results in an elevation of the diaphragm. Episodic or intermittent hypoxia is associated with many pathophysiological situations including sleep apnoeas and apnoeas of prematurity. Chronic intermittent hypoxia leads to systemic hypertension, myocardial and brain infarctions, cognitive dysfunction and sudden death in the elderly (for a review see Prabhakar, 2001; Prabhakar et al., 2001).

On the other hand, in chronic sustained hypoxia (CSH), the low PO_2 exposure lasts for hours to days to months or years. CSH induces gene expression, leading to profound morphological as well as biochemical changes in the CB. It has been demonstrated that chronic sustained hypoxia induces an increase in the sensitivity of CB chemoreceptors to acute hypoxia (Barnard et

al., 1987) and this mechanism plays a significant role in the time-dependent increase in ventilation in breathing on ascent to high altitude, which is termed ventilatory acclimatization to hypoxia (Bisgard 1994, 1995, 2000; Powell et al., 2000a; Powell, 2007). Chronic sustained hypoxia is also involved in pathological conditions, for example in humans CSH is associated with chronic obstructive pulmonary disease (COPD), asthma or pulmonary fibrosis originating pulmonary hypertension, and in infants it is associated with SIDS.

Early theories and experiments on ventilatory acclimatization to hypoxia (VAH) focused on changes in the pH of cerebrospinal fluid as a stimulus for central chemoreceptors. However, time-dependent changes in this parameter do not explain VAH (Forster and Dempsey, 1981), and lead to the idea of neural plasticity i.e. that central nervous system processing of afferent information is enhanced by CSH. Nowadays, it is known that plasticity occurs both in peripheral and central chemoreceptors (for a review see Powell, 2007).

Chronic sustained hypoxia has been shown to increase oxygen sensitivity in the CB, as it increases the CSN discharges in response to hypoxic tests in several species, such as goats (Nielsen et al., 1988), cats (Banard et al., 1987; Vizek et al., 1987), as well as rats (Chen et al., 2002b; He et al., 2005, 2006). This increase is a major factor for the increased hypoxic ventilatory response (HVR) observed with CSH, as shown by Bisgard et al. (1986a, 1986b) in goats, in a preparation where CB are isolated from systemic circulation, and in which 6 hours of isolated CB hypoxia increased ventilation above the acute HVR (Busch et al., 1985), demonstrating that the response is specific to the CB and not due to central chemoreceptors. This plasticity induced by decreased PO_2 in the CB during the first hours explains VAH. Nevertheless, other mechanisms, like the CNS plasticity seen in longer hypoxic exposures, must be involved (Powell et al., 2000b).

Chronic sustained hypoxia does not alter the CB sensitivity to pH in the rat (Banard et al., 1987) or cat (Lahiri and Delaney, 1975a).

1.6.1. Morphological changes in the carotid body induced by chronic sustained hypoxia

Chronic sustained hypoxia induces enlargement of the CB (Lahiri, 2000). Hypertrophy and hyperplasia of CB chemoreceptor cells was observed following CSH (Laidler and Kay, 1978; McGregor et al., 1984; Pequinot et al., 1984; Heath et al., 1985). Animals and humans that permanently live at high altitude have a frequency of chemodectomas (CB tumours) that exceeds that found at sea level. Accompanying the increase in CB size, it appears that the size of dense core vesicles in cytoplasm of chemoreceptor cells is increased (Laidler and Kay, 1978; Pequinot et al., 1984; Pallot et al. 1986), with density being decreased (Laidler and Kay, 1978; Pallot et al. 1986).

Regarding the morphological alterations in blood vessels produced by CSH, it was observed that CSH induces intense CB vasodilatation and formation and enlargement of new vessels, increasing the vascular volume 10 times after 10 days of CSH (Laider and Kay, 1975) (Figure 13).

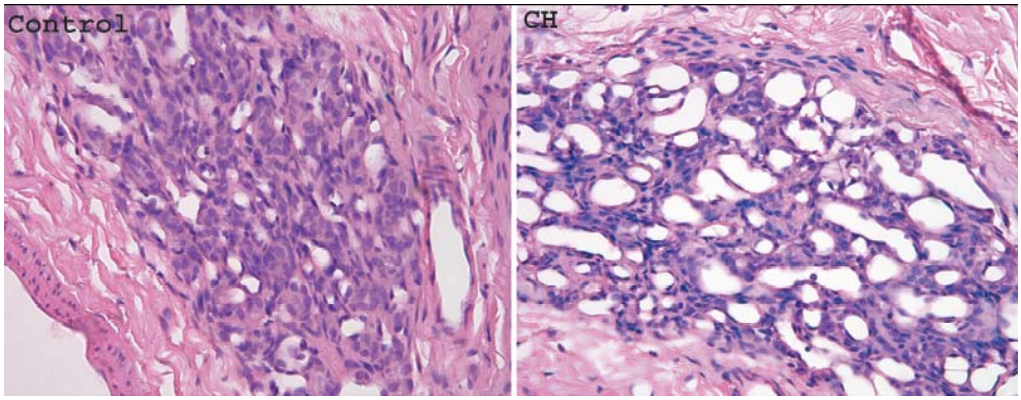


Figure 13 Staining of rat carotid body in control conditions (normoxia – on the left) and when rats were exposed to 1 week of chronic hypoxia. Note that chronic hypoxia induces an increase in the area of the section occupied by vessel lumen in the CB (Wang and Bisgard, 2002).

This increase in vascular volume preferentially involves the large vessels (Clarke et al., 2000). It has been suggested that this increase in vascular volume could be related to an increased expression of vascular endothelial growth factor (VEGF) and thus involves the growth of new vessels, as the VEGF and its receptor Flk-1 are upregulated by CSH. Other authors consider

that CSH does not alter the number of blood vessels but may promote vascular remodelling and proliferation of endothelial cells of the existent vessels (for a review see Wang and Bisgard, 2002).

1.6.2. Neurochemical changes in the carotid body in response to chronic sustained hypoxia

CA are the CB neurotransmitters most studied and well characterized, also having been used several times to assess the function of CB measured as the capacity of chemoreceptor cells to synthesise, store and release CA.

CA metabolism in basal conditions and in response to acute stimulus application, like hypoxia and hypercapnic-acidosis, is extensively described in several mammalian species (Gonzalez et al., 1994; Conde et al., 2006a). In chronic hypoxia some studies have been performed in order to characterize the CA metabolism, but much information about CA metabolism during chronic hypoxia is lacking. Chronic hypoxia increases the level of DA and NE and their turnover rates in the CB (Hanbauer, 1981; Olson et al., 1983; Pequignot et al., 1987; Hui et al., 2003). The increase in the DA content elicited by chronic hypoxia can be seen in the CB after 2 days of hypoxia (Hanbauer et al., 1981). The mRNA coding for TH and the immunoreactivity for the same enzyme are upregulated in CB chemoreceptor cells within the first few hours of hypoxia (Czyzk-Krzeska et al., 1992; Hui et al., 2003). Using techniques of immunocytochemistry (Wang et al., 1998) and RT-PCR (Wang and Bisgard, 2002; Ganfornina et al., 2005) it has also been demonstrated that TH expression and mRNA coding for TH, respectively, increased approximately 3 times in the CB with chronic hypoxia exposure. In addition, it has also been seen that TH activity is increased in chronic hypoxia; in the carotid body (Hanbauer, 1977, Gonzalez et al., 1979a, 1979b, 1981) and in PC12 cells (Millhorn et al., 1997). The fact that chemoreceptor cells cultured in hypoxia during 7 days increase their DA basal release (Jackson and Nurse, 1997) suggests that chronic hypoxia increases DA production and release independently of neuronal or hormonal factors. It is known that D_2 dopamine receptors are present in the CB (Dinger et al., 1981; Bairam and Khandjian, 1997; Gauda et al.1994, 2000) and is thought that they can modulate the

hypoxic ventilatory response. It has been observed that after two days of hypoxia, the mRNA that codes for D_2 receptors decreases. Nevertheless, after 7 days of exposure to hypoxia, the CB D_2 mRNA levels increase (Huey and Powell, 2002). These alterations in the mRNA levels of D_2 receptors are in accordance with the DA-mediated inhibition of ventilatory response induced by chronic hypoxia (Huey et al., 2000).

Acetylcholine is another mediator involved in neurotransmission in the CB and is described as being excitatory in the process of CB chemoreception (Fitzgerald, 2000). It has been described that ACh may regulate the release of DA from CB during chronic hypoxia (Jackson and Nurse, 1998) and that ACh induces a higher response in the CSN chemosensory activity after chronic hypoxia, and that it is almost completely blocked by mecamylamine (He et al., 2005). However, nicotinic and muscarinic receptor antagonists failed to decrease the CSN chemosensory activity induced by acute hypoxia and hypercapnia after chronic hypoxia exposure, suggesting that cholinergic transmission in not involved in acclimatization (He et al., 2005).

As described in section 1.5.4., it has recently been suggested by Zhang et al. (2000) that ATP and ACh are co-released from CB chemoreceptor cells, being the primarily mediators of chemotransmission in the CB. In a recent manuscript by He et al. (2006) it was demonstrated that immunoreactivity to $P2X_2$ receptors in chemoreceptor cells and in PG were marginally elevated and maintained, respectively, after chronic hypoxia exposure. These same authors showed that the effectiveness of P2 receptor blockers in decreasing the CSN chemosensory activity induced by hypoxic tests in chronically hypoxic rats is greater than in normal preparations (when the CSN frequency is expressed as absolute magnitude), suggesting that ATP acting on $P2X_2$ receptors contributes towards adjusting chemoreceptor activity after chronic hypoxia (He et al., 2006). However, when the data are expressed as a percentage of nerve activity, the effect of the P2 antagonists was less effective in chronic hypoxia than in normal preparations (He et al., 2006) indicating that more experiments have to be performed in order to establish the role of ATP in VAH during chronic hypoxia. He et al. (2006) also suggest that, as $P2X_2$ receptor antagonists failed to completely block the acute hypoxia-evoked CSN activity, another neurotransmitter, such as 5-HT, could be involved (He et al., 2006). However, it

has been shown that chronic hypoxia does not modify 5-HT immunoreactivity in the CB (Kameda et al., 1998) and functional studies on the role of 5-HT in hypoxic ventilatory response during chronic hypoxia are lacking.

Adenosine A_{2A} receptors are expressed in rat CB chemoreceptor cells (Gauda et al., 2000) and are up-regulated by chronic hypoxia in PC12 cells (Kobayashi et al., 2000a). It has also been shown that chronic hypoxia enhances adenosine release in PC12 cells by altering adenosine metabolism and membrane transport (Kobayashi et al., 2000b) suggesting that adenosine has a role in chronic hypoxia adaptive responses. Nevertheless, little is known about the functional significance of adenosine in chronic hypoxia in the CB. After chronic exposure to 10% O_2 a moderate down-regulation of adenosine A_{2B} receptor was observed (Ganfornina et al., 2005). Recently, it has been shown that 8-SPT, an antagonist of adenosine receptors, attenuates the increase in respiratory frequency elicited by acute hypoxic tests in rats exposed to an atmosphere of 12% O_2 for 7 days (Walsh and Marshall, 2006). These authors suggested that adenosine in acute hypoxia stimulates A_2 receptors to increase respiration in chronically hypoxic rats.

Also, CB cells exhibit upregulation of angiotensin AT_1 receptor in response to chronic hypoxia, with the additional observation that chronic hypoxia also enhanced AT_1 receptor-mediated excitation of CB afferent activity (Leung et al., 2000) and intracellular Ca^{2+} elevation induced by endothelin-1 and upregulation of endothelin-1 ET_A receptor (Chen et al., 2002a, 2002b). These effects can be important in the modulation of cardiorespiratory function as well as fluid and electrolyte homeostasis and in mitogenesis, respectively, during chronic hypoxia. Another peptide that is present is the CB is SP. However, it is not probable that SP contributes to an increase in the sensitivity of CB to hypoxia, since SP immunoreactivity in chemoreceptor cells is diminished during chronic hypoxia (Wang et al., 1998).

In this work we will focus on the effects of chronic sustained hypoxia in the carotid body and on the role of adenosine in this response. In order to investigate the role of adenosine in this paradigm we have blocked all adenosine receptors using a chronic treatment with caffeine, a non-selective antagonist of adenosine receptors. This has allowed us to study the CB function at several levels: assessing the expression of TH; monitoring the ability of

chemoreceptor cells to store and synthesise CA and release CA, adenosine and ATP; measuring the CSN chemosensory activity in response to different stimuli; and measuring ventilation by whole-body plethysmography.

1.6.3. Chemoreceptor function blunting after prolonged hypoxia

Humans who are native to high altitudes or living at high altitudes for many years may have subnormal or complete loss of the ventilatory response to acute hypoxia, commonly referred to as a "blunted" response to hypoxia (Lahiri et al., 2000). This effect has been attributed to a loss of responsiveness of the CB to hypoxia. Some authors have reported a decreased carotid body hypoxic sensitivity in response to a prolonged exposure to hypoxia (3-4 weeks at an altitude of 5500 m or breathing 10% O_2) that contributes to the decreased hypoxic ventilatory response in chronic hypoxia (Tatsumi et al., 1995a; Wach et al., 1989), this effect being mediated by dopamine, since domperidone and haloperidol lead to increases in CSN chemosensory activity and ventilation (Tatsumi et al., 1995b) in response to acute hypoxia. These authors suggest that this decreased response to acute hypoxia is due to an excess of dopaminergic activity in CB that may interact with ion channels making chemoreceptor cells less excitable (Wyatt et al., 1995; Peers et al., 1996; Carpenter et al., 1998). In fact it was observed by Carpenter et al. (1998) that adult rat dissociated CB chemoreceptor cells undergo selective suppression of Ca^{2+}-insensitive voltage gated K^+ currents in response to a 3 week period of hypoxia (10% O_2) exposure *in vivo*, conditioning the depolarisation of chemoreceptor cells in response to acute hypoxia and the release of neurotransmitters. Wang and Bisgard (2002) have suggested that an excess of CB dopaminergic activity cannot be responsible for the blunting after prolonged hypoxia, because excessive CB dopaminergic activity seems to begin before blunting has developed, at a time when the CB still responds to acute hypoxia. Recently it has been shown that T-type Ca^{2+} channels are up-regulated in hypoxia in PC12 cells and probably in other tissues (Del Toro et al., 2003) and it has been suggested that upregulation of these channels may contribute to cellular functions susceptible to modulation by low O_2 concentrations, like cellular excitability, differentiation, growth and proliferation. Given the broad

distribution of these channels, they could have a major role in cell adaptation to chronic hypoxia (Lopez- Barneo et al., 2004).

1.7. Caffeine

Caffeine is the most widely consumed behaviourally active substance in the world. All caffeine comes from dietary sources, the majority coming from coffee, tea and also from soft drinks and chocolates. Coffee consumption from all sources can be estimated to be around 70-76 mg/person/day in the world, but in countries like the USA, Canada, Sweden, Finland or United Kingdom the values increase (for a review see e.g. Fredholm et al., 1999). Acute and, especially, chronic caffeine intake appears to have minor negative consequences on health. Nevertheless, in the nineties, caffeine was described as a "model drug of abuse" (Holtzman, 1990).

At a cellular level caffeine can act through three distinct mechanisms: 1) releasing Ca^{2+} from intracellular deposits probably via ryanodine receptors, which occurs with millimolar concentrations of caffeine; 2) inhibiting cAMP phosphodiesterases, which occurs with high micromolar concentrations of caffeine, higher than those attained with standard human caffeine consumption; and 3) inhibiting adenosine receptors and antagonising of the actions of adenosine, the natural agonist of these receptors, this being the only mechanism significantly affected with the standard doses of human caffeine consumption (Fredholm et al., 1999, Figure 14).

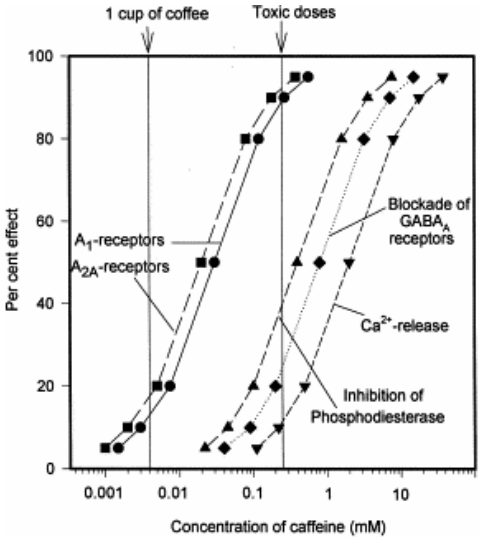


Figure 14 Caffeine effects on different biochemical targets in relation to its levels in humans. Note that caffeine can inhibit adenosine receptors after a single cup of coffee. To inhibit cAMP phosphodiesterases a concentration 20 times higher is required, and to mobilize Ca^{2+} from the intracellular deposits a concentration 100 times higher is required. (Fredholm et al., 1999)

Of the four adenosine receptor subtypes, caffeine and theophylline are most potent at A_{2A} receptors, followed closely by A_1 receptors and then A_{2B} receptors in humans (Klotz et al., 1997) (Table 3). At human A_3-receptors caffeine is a weak antagonist. Since caffeine is an antagonist, not an agonist, at adenosine receptors, one would expect no pharmacological effects unless the receptor is being activated by endogenous adenosine.

Some years ago it was thought that concentrations of adenosine existent in physiological conditions only activate A_1 and A_{2A} receptors, A_{2B} (or A_3) being activated in pathophysiological conditions, such as ischaemia or convulsions, when adenosine levels are higher. For this reason, during many years it was thought that blockade of A_1 and A_{2A} receptors could be the most likely mechanism of action for caffeine under normal physiological conditions in the brain.

Table 3 Potency of caffeine at rat and human adenosine receptor subtypes.

Receptor subtype	Rat (Kd) μM	Human (Kd) μM
A_1	20	12
A_{2A}	8.1	2.4
A_{2B}	17	13
A_3	190	80

Nevertheless, Fredholm (2006, personal communication) recently suggested that the activation of adenosine receptors does not depend only on the concentrations of adenosine, but also on the density of the receptor subtypes existent in the site of action. This means that caffeine can also block A_{2B} adenosine receptors in physiological concentrations.

1.7.1. Effects of acute caffeine treatment on ventilation

It is known that methylxanthines, particularly caffeine, stimulate ventilation and decrease apnoeic episodes in premature infants (Aranda and Turmen, 1979; Bairam et al., 1987), caffeine being a drug of choice to treat the

apnoea of prematurity (Comer et al., 2001). This analeptic effect of caffeine is due to the blockade of inhibitory A_1 adenosine receptors in central respiratory neurons (Gautier and Bonora, 1982; Mazzarelli et al., 1986; Herlenius and Lagercrantz, 1999). However, Wilson and co-workers (2004) have recently demonstrated the modulation of the GABAergic inputs affecting respiratory timing and inspiratory drive in the medulla oblongata of neonatal piglets by A_{2A} adenosine receptors. Yet, adenosine at the peripheral chemoreceptors is a stimulating agent (see section 2.1.4.) and in un-anaesthetised adult monkeys, caffeine significantly attenuated the CB-mediated hyperventilation occurring while the animals were breathing 10% O_2 (Howell and Landrum, 1995). It was also observed that bolus caffeine infusion induces a rapid increase in ventilation that is abolished with peripheral chemodenervation (Blanchard et al., 1986) and that administration of aminophylline after carotid body chemodenervation does not prevent the profound respiratory depression elicited by hypoxia in chemodenervated piglets (Cattarossi et al., 1995). In any case, these last findings suggest that endogenous adenosine contributes to the activation of the CB chemoreceptors during hypoxia. However, Bairam et al. (1997) concluded that, although caffeine stimulates CSN discharge, the effect is short lived and appears not to be dependent on dopaminergic mechanisms or adenosine A_2-receptor antagonism and suggested that the effect on CSN is through a modification of carotid chemoreceptor blood flow associated with the change in blood pressure.

These findings on the effects of caffeine on peripheral control of breathing are contradictory and the identity of the adenosine receptors involved in adenosine and caffeine effects on CB chemoreceptors remains to be established.

1.7.2. Chronic treatment with caffeine

Adaptive changes to chronic caffeine treatment are very dramatic, being not only quantitatively different from but often opposite to the acute effects of caffeine on normal and pathological conditions.

Chronic treatment with caffeine can result in alterations in adenosine receptors and other receptors. “Up-regulation” of A_1-adenosine receptors,

serotonin receptors, $GABA_A$ receptors, muscarinic receptors and δ-opioid receptors and the ”down-regulation” of β-adrenergic receptors and ‘desensitization' of nicotine receptors occurs after chronic caffeine in the cortex and striatum of mice (Shi and Daly, 1999). An increased density of L-Type channels (Shi and Daly, 1999) has also been observed. In the striatum in the rat, a down-regulation of A_{2A} adenosine receptors (Svennigsson et al., 1999) has been seen, but in human platelets chronic caffeine intake “up-regulates” A_{2A} receptors, this effect being accompanied by sensitisation to HE-NECA, a selective A_{2A} adenosine receptor agonist (Varani et al., 1999). It has also been observed that chronic caffeine intake down-regulates the metabotropic glutamate receptors in the rat heart (Iglesias et al., 2006). Such alterations might be relevant to chronic consumption of caffeine in humans and might in humans, as in mice, affect responses to agents acting through such receptors. However, these adaptive changes do not indicate what the primary targets are during acute treatment with caffeine. For example, dopamine receptors appear to be unaffected by chronic caffeine (Shi et al., 1993, 1994), but considerable evidence suggests a role for dopaminergic systems in the positive effects of caffeine in humans and animals (see Ferré et al., 1997; Garrett and Griffiths, 1997). Recently, in other preparations, PC12 cells and striatal cultures, it was observed that chronic treatment with caffeine stimulates D_2 mRNA expression, these effects being abolished by treatment with CGS21680, an A_{2A} agonist (Stonehouse et al., 2003) suggesting that, in part, the effects of caffeine on D_2 receptors are mediated via A_{2A} receptors. In pathological conditions, a correlation between caffeine intake and the increased adrenergic tone (increased NE levels) in humans with sleep apnoea is also described (Bardwell et al., 2000).

Long-term treatment with caffeine decreases locomotor activity (Nikodijeviç et al., 1993) while an acute treatment stimulates locomotor behaviour in rodents. In the same way, caffeine long-term treatment leads to an improved spatial learning capacity and acute treatment does not. Chronic administration of caffeine (or theophylline) leads to significant protection against seizures, whether they are induced by NMDA or by $GABA_A$ receptor antagonists. These data reflect the fact that the effect of chronic caffeine is not

related to any specific seizure, but is more general, occurring without any change in A_1 receptors (Georgiev et al., 1993; Jacobson et al., 1996).

In pathological conditions, for example in ischaemia, long-term caffeine treatment leads to a decreased susceptibility to ischemic brain damage (Rudolphi et al., 1989) and acute treatment exacerbates the damage (Dux et al., 1990).

All these results indicate that the long-term treatment with caffeine in doses similar to those habitually consumed by humans can induce adaptive changes in the brain and possibly in the whole body.

Nothing is known about the chronic effects of caffeine on the activity of CB chemoreceptors. This point can be important because patients with obstructive sleep apnoea (OSA) are use high amounts of caffeine to fight sleepiness and caffeine could modify chemoreflexes in OSA and thereby increase or decrease the sensitivity of CB to hypoxia.

2. GENERAL AND SPECIFIC AIMS

The general aim of this PhD thesis was to establish the functional significance of adenosine in chemoreception at the carotid body in control and chronically hypoxic rats.

To achieve this objective we investigated:

1) The release of adenosine from a rat carotid body *in vitro* preparation in response to moderate hypoxia and the specificity of this release. We also investigated the metabolic pathways of adenosine production and release in the organ in normoxia and hypoxia;

2) The modulation of adenosine/ATP release from rat carotid body chemoreceptor cells by nicotinic ACh receptors;

3) The effects of caffeine on peripheral control of breathing and the identity of the adenosine receptors involved in adenosine and caffeine effects on carotid body chemoreceptors;

4) The interactions between dopamine D_2 receptors and adenosine A_{2B} receptors that modulate the release of CA from the rat carotid body;

5) The effect of chronic caffeine intake i.e. the continuous blockage of adenosine receptors and thereby simulating a caffeine dependence, on the carotid body function in control and chronically hypoxic rats.

3. CHAPTER 1 – STUDY OF THE EFFECT OF HYPOXIA ON THE RELEASE OF ADENOSINE FROM THE RAT CAROTID BODY

3.1 Introduction and aim

As described in section 1.5.1.4. of the general introduction, excitatory effects of adenosine on CB chemosensory activity have been described. The role of adenosine in chemoreception will be reinforced if this mediator is involved in the specificity of the response of CB chemoreceptors to acute hypoxia. Knowing that the threshold tissue PO_2 for carotid sinus nerve discharge to increase oscillates between 55-65 mmHg, which corresponds to arterial blood PO_2 values of 70-75 mmHg (Obeso et al., 1997), we hypothesise that arterial chemosensitivity could be associated with a low threshold to released adenosine in response to hypoxia. In order to study the specificity of the release of adenosine from CB chemoreceptor cells by hypoxia, other tissues, namely SCG and arterial tissue, were used as controls. In the current chapter, we have also investigated the metabolic pathways of adenosine production and release in the organ.

3.2. Material and methods

3.2.1. Tissue preparation and experimental conditions

Wistar rats (200-300 g) from the Faculty of Medical Sciences animal house kept at a constant temperature (21ºC) and a regular light (08.00-20.00h) and dark (20.00-08.00h) cycle, with food and water *ad libitum* were used throughout the experiments. The rats were anaesthetized with sodium pentobarbital administered intraperitoneally (60 mg/kg, i.p.). After tracheostomy, CB, SCG and common carotid artery bifurcations were removed under a Nikon SMZ-2B dissection scope and placed in 500 μL of ice-cold 95% O_2 + 5% CO_2 equilibrated incubation medium containing different drugs in accordance with the protocol used. The incubation medium was a modified Krebs solution (Perez-Garcia *et al*., 1990) composed of (mM): NaCl 116; $NaHCO_3$ 24; KCl 5; $CaCl_2$ 2; $MgCl_2$ 1.1; HEPES 10; glucose 5.5; pH 7.42. Due to the small dimensions of the CB (≈ 50 μg) four CBs were used in each experiment. In all experiments, before the incubation periods, the CBs, SCG and common carotid

artery bifurcation samples were submitted to a 30 min period of pre-incubation in hyperoxia (95% O_2 + 5% CO_2) at 37º C. This allowed recovery from the ischemia to which these preparations were submitted when removed (see e.g. Perez-Garcia et al. 1990). The experiments were performed under either hypoxic or normoxic conditions, meaning that tissues were incubated in media equilibrated with 10% O_2 : 5% CO_2 : 85% N_2 or 20% O_2 : 5% CO_2 : 75% N_2 respectively. After removal of the tissues, the animals were killed by an intracardiac injection of a lethal dose of pentobarbital in agreement with the directives of the European Union (Portuguese law nº 1005/92).

3.2.2. Characterization of the content and release of adenosine in rat carotid bodies

The CBs were incubated for either 10 or 30 min under hypoxic or normoxic conditions in medium containing an inhibitor of adenosine deamination, EHNA (2.5 μM). After incubation the CBs were removed from the medium, and the amount of adenosine released was measured. CBs were further used to quantify adenosine content. For these determinations CBs were submitted to a 30 min period of normoxic or hypoxic incubation. After incubation the CBs were removed from the incubation medium and were immersed in ice-cold perchloric acid (3M during 10 min), homogenized in an Ultra-Turbax (IKA Laborthecnik, Staufen, Germany) and maintained at 0º C for 30 min. The samples were further centrifuged at 12000 g for 5 min (4º C) and adenosine was extracted from the supernatant.

3.2.3. Characterization of the release of adenosine from SCG and arterial tissue

For characterization of adenosine released from these tissues two parameters were studied, the time dependence on this release and the effect of different percentages of oxygen (2%, 5% and 10%). For the temporal study on adenosine release, SCG and arterial tissue were submitted to an incubation period of 10 or 30 min in 10% O_2 or normoxia in a medium containing EHNA (2.5 μM). The effects of different percentages of oxygen on the release of

adenosine were studied incubating SCG and arterial tissue during 10 min in a incubation medium containing EHNA (2.5 μM) and equilibrated with 2%, 5% and 10% O_2 plus 5% CO_2. After the incubation period the tissues were weighed and the nucleotides released were extracted from the incubation medium.

3.2.4. Effect of adenosine transporter inhibitors

Two inhibitors of the ENT system were used in these experiments: dipyridamole and NBTI (Dunwiddie and Diao, 1999; Leung et al., 2001). The CBs were incubated for 30 min under either hypoxic or normoxic conditions in medium containing EHNA (2.5 μM) plus dipyridamole (50 μM) or NBTI (5 μM). After the incubation period the CBs were removed and the nucleotides extracted from the medium.

3.2.5. Metabolic pathways of adenosine production at the rat carotid body

The metabolic pathways of adenosine production were studied in the presence of inhibitors of adenosine catabolism and transport, and inhibitors of the ecto-5´-nucleotidases. Experiments were carried out with CBs incubated for 30 min in normoxic or hypoxic conditions: in the presence of EHNA (2.5 μM) only; in the presence of EHNA (2.5 μM) and NBTI (5 μM); in the presence of EHNA (2.5 μM) and AOPCP (100 μM) – inhibitor of ecto-5´-nucleotidases and in the presence of EHNA (2.5 μM), NBTI (5 μM) and AOPCP (100 μM). After the incubation period the CBs were removed and adenosine extracted from the incubation medium.

3.2.6. Nucleotide extraction

Nucleotides were extracted from the incubation medium following a previously described protocol (Cunha et al., 1994) and aliquots of the neutralized supernatants were collected and kept at –20º C until subsequent analysis by high-performance liquid chromatography (HPLC).

3.2.7. HPLC analysis

The HPLC system consisted of an LC 9-A solvent delivery pump (Shimadzu Corporation, Kyoto, Japan), an 7725i injector (Shimadzu Corporation, Kyoto, Japan), an SPD-6 AV UV-VIS wavelength detector (Shimadzu Corporation, Kyoto, Japan) and Shimadzu Class VP software to analyse the chromatograms. The analytical column was a Lichrospher 100 RP-18 (125×4 mm, I.D, particle size 5 μm, Merck) protected by LichroCART 4-4 guard-columns (Merck). The columns and guard-columns were incorporated into the HPLC system through a ManuCART (Merck). Isocratic elution was used: the mobile phase consisted of a solution of KH_2PO_4 100 mM with 15% of methanol, pH 6.5 run at a flux of 1.75 mL/min. This method for adenosine quantification was adapted from that described by Cunha et al. (Cunha et al., 1989). Standards were prepared under the same conditions as the biological samples.

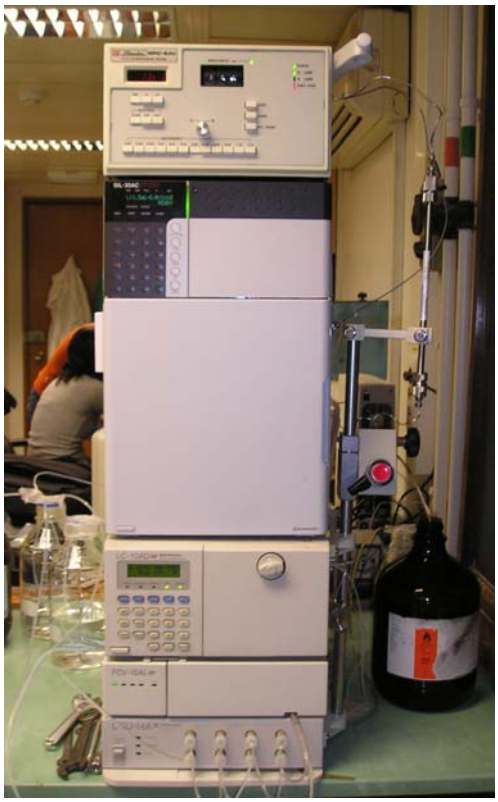


Figure 15 HPLC setup constituted of, from the bottom to the top, a degasser, solvent mixer, pump, automatic injector and a UV-detector. At the right of the HPLC tower components, we can observe a manual injector.

Three calibration curves performed in triplicate were used. The identification of the peaks in the biological samples was made by comparison

with the retention times of the standards. Chromatograms of the incubation medium with EHNA and dipyridamole or NBTI were also obtained under the same conditions in order to identify the peaks of these drugs in the chromatograms. To confirm the presence of adenosine in the chromatograms, experiments were performed in which the CBs were incubated with adenosine deaminase (EC 3.5.4.4, 2 U/mL, Sigma) in normoxia, then submitted to the same experimental conditions described above.

Cellular viability of the CBs in hypoxia and normoxia was assessed measuring lactate dehydrogenase (EC 1.1.1.27) activity at 365 nm in samples incubated under the same conditions used for the quantification of adenosine released by CBs, using a standard optimised commercial kit (MPR1, Roche Sistemas de Diagnóstico, Linda-A-Velha, Portugal).

Protein concentration was quantified (Peterson, 1977) in the samples, CBs and incubation medium after nucleotide extraction, to confirm the absence of proteins before HPLC analysis.

3.2.8. Drugs

Adenosine, sodium pentobarbital, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), dipyridamole, α,β-methylene ADP (AOPCP), S-(p-nitrobenzyl)-6-thioinosine (NBTI), adenosine deaminase were all from Sigma (St Louis, MO, USA). Dipyridamole and NBTI were dissolved in dimethylsulphoxide in a 2 and 10 mM stock solution, respectively.

3.2.9. Data analysis

The amount of adenosine released by the CBs and the adenosine content of the CB content, quantified by HPLC, was expressed in pmol/CB after division of the absolute values obtained from the chromatograms by four (four CBs were used in each incubation) and correction for the volume used. The weight of CBs in adult rats varies between 40-55 μg (McDonald, 1980, Conde et al., 2006a), and adenosine concentrations in the present work were around 100 pmol/CB. The variability in CB weight introduces a maximal error of 1.5 pmol if expressed in pmol/mg tissue. Adenosine values released by SCG and arterial

tissue were expressed in pmol/mg of tissue. Data were evaluated using Graph Pad Prism software, version 3 and were presented as mean ± SEM values. The significance of the differences between the groups means were calculated by the unpaired Student's *t* test and by one-way analysis of variance (ANOVA) with Dunnett´s multiple comparison test. Values of $P < 0.05$ were considered as representing significant differences.

3.3. Results

3.3.1. Adenosine quantification

The chromatograms corresponding to standard samples of adenosine (3-5000 nM) used in the preparation of calibration curves showed concentration-dependent peaks with retention times of 2.25 ± 0.04 min (n = 33). Figure 16A represents an example of adenosine chromatograms obtained with four different concentrations inside the range used for calibration. In order to verify whether these peaks correspond to adenosine, chromatograms of the incubation medium of CBs containing adenosine deaminase (2 U/mL) were performed. In these chromatograms the peaks corresponding to the retention time of adenosine were abolished (Figure 16B).

Calibration curves showed a linear relationship ($r > 0.99$) between the area of the peak and the concentration of adenosine injected (3-5000 nM). The limit of detection, defined as the lowest concentration of a test substance that the analytical process can reliably differentiate from background levels (Shah *et al*., 1991), was 10 nM. The limit of quantification, defined as the lowest concentration that can be measured with a stated level of confidence (Shah *et al*., 1991), was 0.2 pmol/mL.

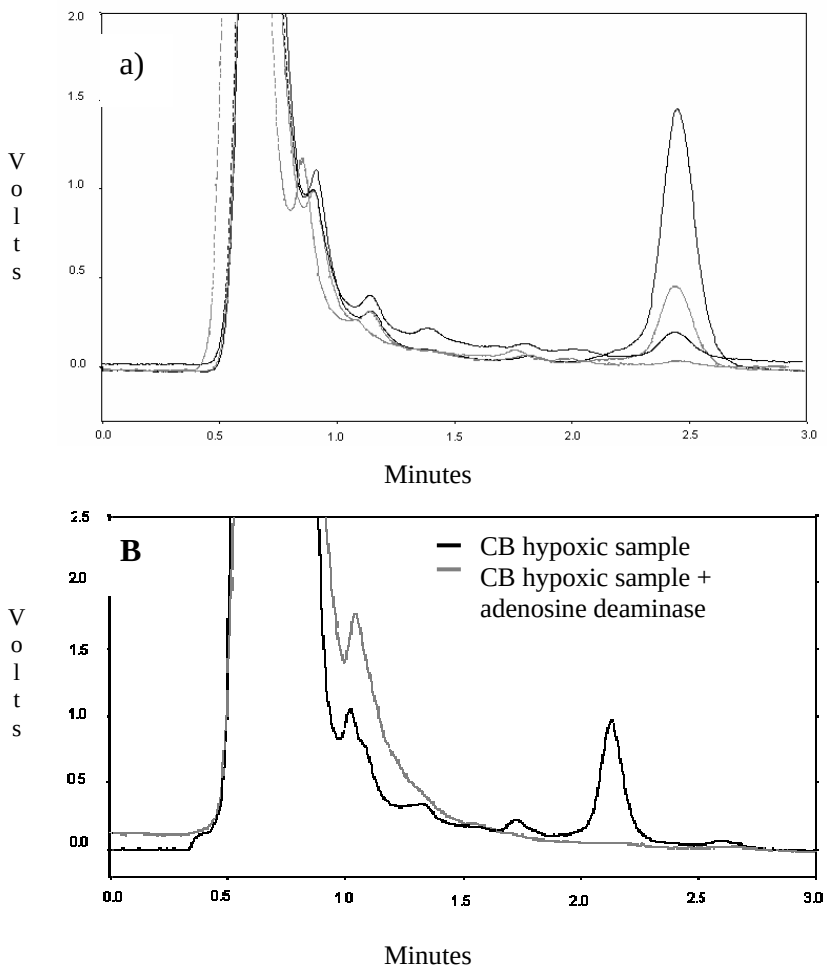


Figure 16 Chromatograms of adenosine obtained by reverse phase HPLC. (a) Juxtaposition of the chromatograms obtained with four different concentrations of standard adenosine solutions (30, 300, 1000 and 3000 nM) in incubation medium with EHNA (2.5 μM); (b) Juxtaposition of the chromatograms of adenosine released by carotid bodies submitted to hypoxia (10 % O_2, 30 min) in the absence (▬) and in the presence (▬) of adenosine deaminase (2 U/mL).

3.3.2. Adenosine content and release from carotid bodies during hypoxia

Adenosine has a short half-life (<10 s) (Moser *et al.*, 1989) and is converted by adenosine deaminase (EC 3.5.4.4) to inosine. To avoid the degradation of adenosine released from the CB, the adenosine deaminase inhibitor EHNA (2.5 μM) was present throughout the experiments. Adenosine was detected in the incubation medium during normoxia, and hypoxia caused a statistically significant increase in release of the nucleoside (Fig. 17A). The accumulation of adenosine released by the CB during hypoxia was time-dependent (Figure 17A) ($P < 0.05$, adenosine concentrations released by CBs during 30 min versus 10 min). The increases in adenosine release during 10 and 30 min of hypoxic incubation were 34.1% and 44%, respectively (Figure 17A).

The effect of hypoxia on the adenosine content of the CBs was also quantified. After 30 min of exposure to hypoxia a dramatic reduction in adenosine content was observed (Figure 17B).

Analysis of cellular viability of the CBs in hypoxia and normoxia has showed that although they were both in the reference domain (< 240 U/L), i.e. above the allowed limit of cellular death, cellular viability in normoxia (42.85 U/ L) was higher than in hypoxia (127.7 U/ L).

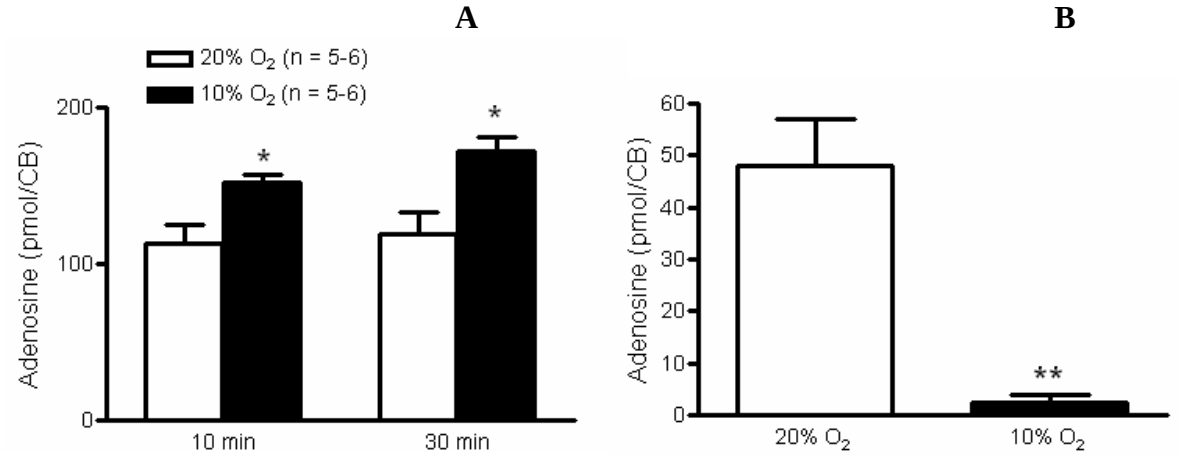


Figure 17 Effect of hypoxia (10% O_2) on adenosine levels (A) released by the carotid bodies (CBs) and (B) quantified in the CBs after the incubation period. **A**) Effect of two different periods of incubation, 10 min and 30 min, on the release of adenosine by the CBs. **B**) Adenosine content in CBs after an incubation period of 30 min (n = 9-12). All the experiments were performed in the presence of EHNA (2.5 μM) and 5% CO_2. * P < 0.05; unpaired Student's t test corresponding to adenosine concentrations released by CBs in normoxia (20% O_2) versus hypoxia. ** P < 0.0001; unpaired Student's t test corresponding to the differences between the content of adenosine in the CBs after normoxia and hypoxia. Vertical bars represent mean ± SEM.

3.3.3. Adenosine released by SCG and arterial tissue

The effect of different percentages of oxygen (2, 5 and 10%) and different times of CB incubation on the release of adenosine was also tested in the same experimental conditions in other preparations: arterial tissue (common carotid arteries) and SCG. In the presence of EHNA (2.5 μM), adenosine release during 10 min by SCG was about 3-4 times higher than that released from arterial tissue (Figure 18). After 10 min of exposure to 10% O_2, the amount

of adenosine released by the SCG and arterial tissue did not change significantly ($P > 0.05$) (Figure 18).

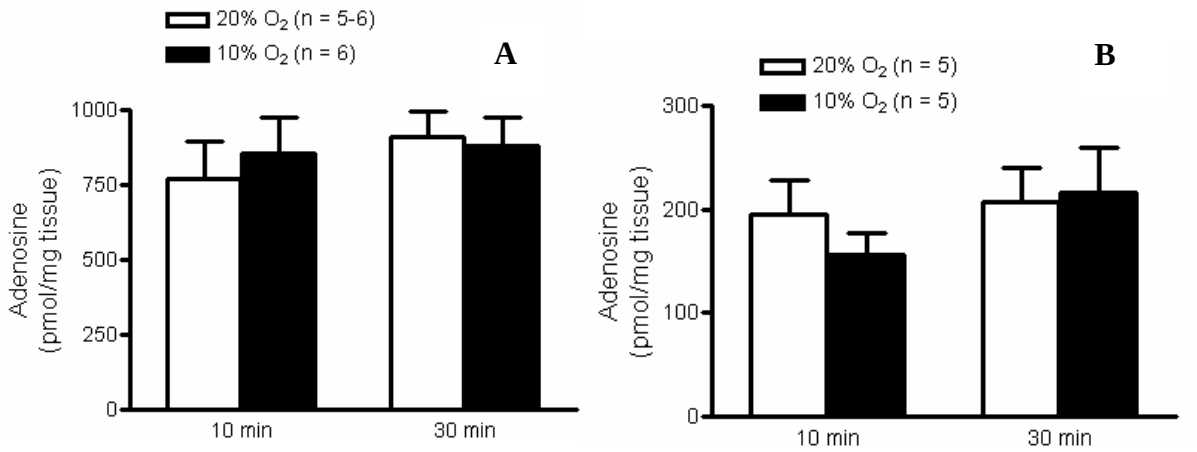


Figure 18 Adenosine released from (**A**) rat superior cervical ganglions (SCG) and (**B**) arterial tissue (common carotid artery bifurcations) during 10 and 30 min periods of normoxia (20% O_2) and hypoxia (10% O_2). All the experiments were performed in the presence of EHNA (2.5 μM) and 5% CO_2. No significant statistical differences ($P > 0.05$; unpaired Student's *t* test) were observed between adenosine released from the tissues in the two different incubations times, nor between normoxia and hypoxia. Vertical bars represent mean ± SEM.

Incubation of the CBs during longer periods of moderate hypoxia (30 min) did not modify adenosine concentrations released in normoxic and in hypoxic conditions (Figure 18).

After 10 min of exposure to more intense hypoxia, 5% and 2% O_2, adenosine release from SCG and arterial tissue was enhanced (Figure 19).

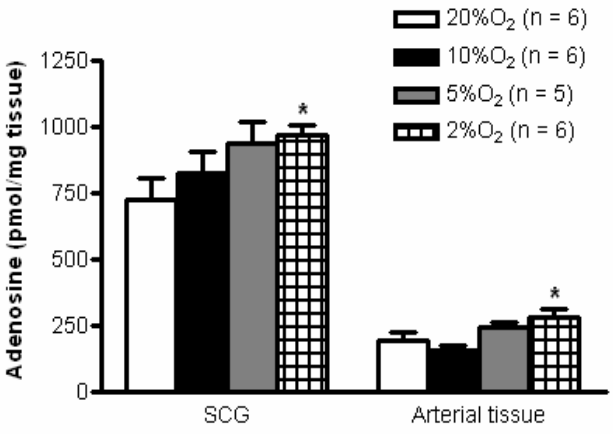


Figure 19 Adenosine released by superior cervical ganglia (SCG) and arterial tissue (common carotid arteries), in response to different percentages of oxygen. All the experiments were performed in the presence of EHNA (2.5 μM) and 5% CO_2 during incubation periods of 10 min. * $P < 0.05$; ANOVA with Dunnett's multiple comparison test between adenosine concentrations released in 20% O_2 and other percentages of oxygen. Vertical bars represent mean ± SEM.

The increases in adenosine achieved during 5% O_2 was not statistically significant (*P > 0.05*) in either tissue, but in response to 2% O_2 statistically significant increases of 44.8% in SCG and 44.4% in arteries were observed (Figure 19).

3.3.4. Effect of adenosine transport inhibitors

The effect of moderate hypoxia (10% O_2) on the release of adenosine from CBs was studied in the presence of two equilibrative nucleosides transport system inhibitors, dipyridamole and NBTI. As illustrated in Figure 20, the increase in adenosine concentrations induced by hypoxia (30 min) remained when nucleoside transport was inhibited. The presence of dipyridamole (50 μM), or NBTI (5 μM) did not significantly modify the amount of adenosine released from CBs either in normoxic or hypoxic conditions (Figure 20).

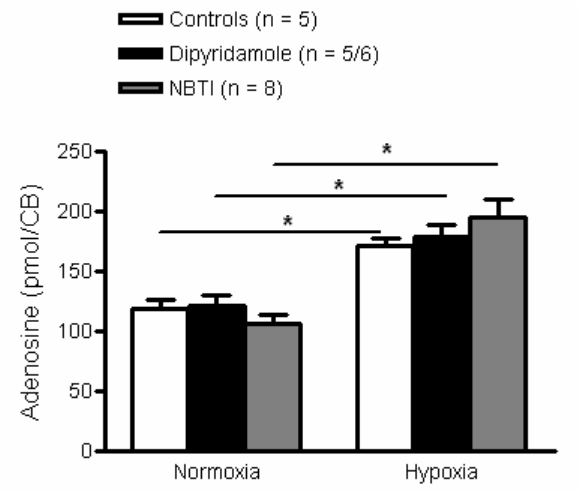


Figure 20 Effects of nucleoside transport inhibitors, dipyridamole (50 μM) and NBTI (5 μM), on adenosine released by carotid bodies (CBs) in normoxia (20% O_2) and hypoxia (10% O_2). In all experiments the CBs were submitted to 30 min incubation periods in the presence of EHNA (2.5 μM) and 5% CO_2. * $P < 0.05$ hypoxia vs. normoxia (ANOVA with Dunnett's multiple comparison test). Vertical bars represent mean ± SEM.

3.3.5. Metabolic pathways of adenosine production at the rat carotid body

The contribution of two adenosine production pathways – ATP extracellular catabolism and specific release of adenosine through the equilibrative nucleoside transport system – to adenosine released by the CB during normoxia and hypoxia (10% O_2) were investigated. Experiments were performed in the presence of the inhibitor of ecto-5'-nucleotidase, AOPCP, alone or plus NBTI (Figure 21). In normoxia (Figure 21A), NBTI (5 μM) alone neither modified ($P > 0.05$) adenosine concentrations (as previously shown in

Figure 20) nor did it modify the effect of AOPCP (100 μM). In contrast, AOPCP (100 μM) reduced adenosine release from the CBs by 44.7% (P < 0.01) in normoxia (Figure 21A) and by 43.7% in hypoxia (Figure 21B).

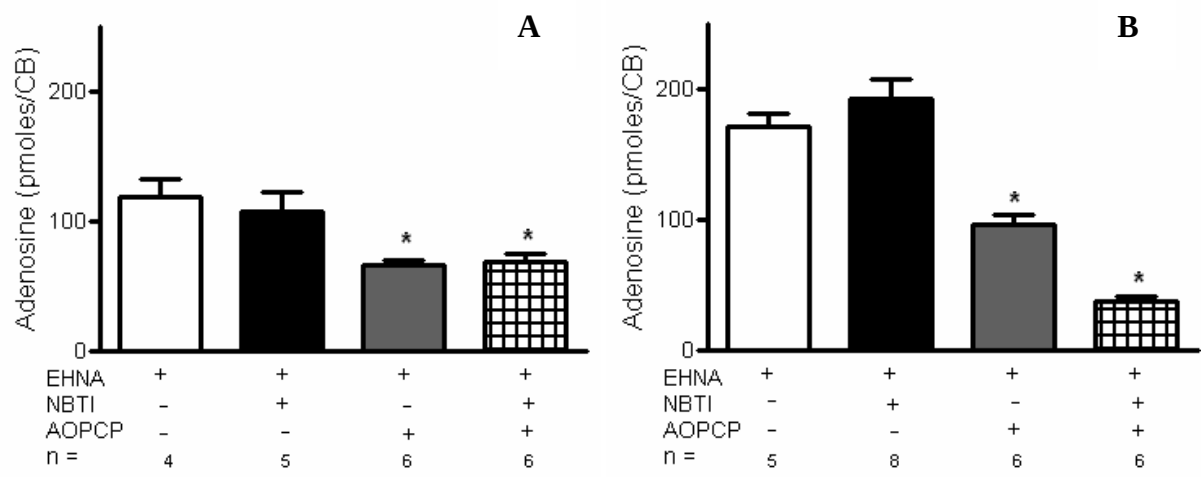


Figure 21 Effects of an inhibitor of nucleoside transport systems, NBTI (5 μM), and an inhibitor of ecto-5'-nucleotidase, AOPCP (100 μM), on adenosine release by carotid bodies (CBs) (**A**) in normoxia (20 % O_2) and **(B)** hypoxia (10 % O_2). The CBs were incubated for 30 min in the presence of 5 % CO_2. * P < 0.01; ANOVA with Dunnett's multiple comparison test between drug treated groups and control values (adenosine released in the presence of EHNA 2.5 μM). Vertical bars represent mean ± SEM.

During hypoxia, when the extracellular pathway of ATP catabolism was intact, NBTI (5 μM) did not modify adenosine concentrations released by the CB (Figs. 20 and 21B). However, in the presence of AOPCP, NBTI caused a further decrease in adenosine concentrations (Figure 21B). Inhibition of the two pathways of adenosine production at the CB with AOPCP plus NBTI reduced the concentrations of adenosine released in response to hypoxia by 78% (P < 0.01) (Figure 21B).

3.4. Discussion

The current study is the first to quantify the release of adenosine from the CB during hypoxia. Hypoxia (10% O_2) induced the release of adenosine from

rat CBs, but was not a powerful enough stimulus to evoke the release of adenosine from SCG or carotid arterial tissue.

Because neither the vascular nor the autonomic component of the CB were as sensitive to oxygen levels as the whole CB, we can postulate that CB-chemosensitive cells are probably mainly responsible for adenosine production in response to moderate hypoxia. Accumulation of extracellular adenosine in response to hypoxia (<10% O_2) has been shown in PC12 oxygen-sensitive cells (Kobayashi *et al*., 2000b).

In this study we did not measure CB tissue PO_2. However, considering that tissue PO_2 values obtained in CBs perfused with air-equilibrated saline (≈ 20% O_2, ≈ 133 mmHg PO_2) presented a mean value of 113 mmHg, we can assume that 10% O2 (≈ 66 mmHg) may determine mean tissue PO_2 values of approximately 55 mmHg, consistent with the threshold tissue PO_2 (55-65 mmHg) for carotid sinus nerve discharge (Obeso et al., 1997b).

Comparison among the absolute values of adenosine produced by the CBs, SCG and carotid bifurcations revealed that the CB produced more adenosine (≈ 100 pmol/CB, corresponding to 3nmol/mg tissue for a 50 µg CB) than the SCG (≈ 800 pmol/mg tissue) or the arteries (≈ 200 pmol/mg tissue).

The existence of adenosine in normoxic conditions can be explained because the rate of adenosine production is higher than its degradation. In the classical works of Schrader and co-workers, particularly in the study describing the short half-life of adenosine in human and dog plasma, a large turnover of plasma adenosine (0.5-1.5 nmol/min ml/blood) was also calculated (Moser et al., 1989).

In basal conditions, adenosine can be produced by intra- and extra-cellular degradation of ATP. It is consensual that physiological levels of adenosine are in the range 0.1-1 µM and they have always been quantified in basal conditions in different preparations (Phillis et al., 1989; Fowler, 1993; Cunha, 1997; Jin and Fredholm 1997; Saito et al., 1999; McLean et al., 1998; Dale et al., 2000). Moreover, degradation of adenosine in *in vitro* preparations and in the presence of EHNA (current work) is slower than in the presence of blood and preserved adenosine deaminase activity.

The amount of adenosine released by CBs, SCG and arterial tissue showed a clear linear correlation with the duration of the hypoxic exposure: higher concentrations were observed after 30 min than after 10 min. However, after 30 min of hypoxia (10% O_2) almost no adenosine was detected in rat CBs. Interestingly, the amount of adenosine released into the incubation medium during 30 min of 10% O_2 and the reduction in adenosine content measured in the same CBs were similar (≈ 50 pmol/CB).

Depletion of adenosine from the CB by hypoxia may be compatible with an initial acute increase of intracellular adenosine due to ATP degradation followed by a massive release of adenosine by the transporters and the incapacity to replenish its source, ATP. However, to confirm this mechanism, kinetic studies on ATP degradation and adenosine production should be done in this preparation.

The amount of adenosine released by the CBs during normoxia and hypoxia was not modified by the two ENT inhibitors, dipyridamole and NBTI, used in supramaximal concentrations (Clanachan et al., 1987; Lynge et al., 2001), but was reduced by the blockade of ATP extracellular catabolism with AOPCP. It was apparent that, at the CB, approximately 40% of extracellular adenosine came from the extracellular catabolism of ATP. The relative contribution of the extracellular catabolism of ATP to the total extracellular levels of adenosine was similar in both normoxic and hypoxic conditions, although more adenosine was produced during hypoxia.

Equilibrative nucleoside transporters are bi-directional (Cass et al., 1998) and the absence of effects of NBTI and dipyridamole during normoxia and hypoxia suggest that the transporter is in equilibrium or does not play a significant role in the maintenance of extracellular adenosine concentrations in basal conditions.

The former interpretation is more probable because equilibrative nucleoside transporter proteins were cloned and functionally characterised in the rat (Yao et al., 1997) and the accumulation of extracellular adenosine during the 30 min in the presence of an inhibitor of its degradation (EHNA) facilitates its uptake. Another finding that supports this mechanism is that when AOPCP was added to the medium, the contribution of the ENT to adenosine production

at the CB was clearly different in normoxia and hypoxia. In these experiments, in which extracellular ATP did not contribute to adenosine production and extracellular adenosine concentrations were lower, the transporter apparently remained in equilibrium while normoxic conditions maintain the balance between ATP synthesis and its catabolism. However, under hypoxic conditions intracellular degradation of ATP clearly caused adenosine release despite its extracellular accumulation.

The effects of NBTI on hypoxia-induced release of adenosine have not previously been described, but enhancement of hypoxia-evoked release of adenosine by the other ENT blocker, dipyridamole, was observed *in vivo* in the rat cerebral cortex (Phillis et al., 1989) and *in vitro* in the rat hippocampal slice (Fowler, 1993). In the current CB preparation, dipyridamole was not used in the experiments with AOPCP because some controversy exists concerning its effects in the rat: dipyridamole does not inhibit rat nucleoside transport proteins *in vitro* (Yao et al., 1997) but modifies adenosine concentrations *in vivo* (Monteiro and Ribeiro, 1989; Phillis et al., 1989) or in *in vitro* functional studies in the rat (Fowler, 1993).

A significant amount of extracellular adenosine was detected in the CB when both ENT and ecto-5'-nucleotidase were inhibited. No dose-response curves for AOPCP or NBTI were performed, but the concentrations used were 1000 times the IC_{50} for ENT1 (Yao et al., 1997) and 10 times higher than the concentration that inhibits 90% of AMP hydrolysis by the ecto-5'-nucleotidase (Meghji and Burnstock, 1995). It is probable that other mechanisms, such as inhibition of adenosine deaminase and *S*-adenosyl homocysteine hydrolysis, are involved in adenosine production and release by the CB in normoxia and hypoxia.

One of the main findings of the present work was the role of the NBTI-sensitive ENT during hypoxia. In addition to extracellular adenosine production that occurs equally in normoxia and hypoxia, low PO_2 triggers adenosine efflux through activation of NBTI-sensitive ENT. This finding was predictable because most studies in the central nervous system demonstrate intracellular formation of released adenosine under ischemic or hypoxic conditions (for a review see Latini and Pedata, 2001). In these *in vitro* studies hypoxic conditions include

perfusions with 0% O_2. Therefore, the comparison of the pathways of adenosine production in vascular tissue and SCG did not seem to be relevant.

In the CB, the finding that hypoxia activates adenosine release *per se* supports the hypothesis that adenosine is one of the important excitatory mediators that increase ventilation via activation of through carotid sinus chemosensory nerves. This work also confirms ATP as an important source of extracellular adenosine production, although the contribution to adenosine production is similar in normoxia and in hypoxia. ATP produced at the CB can activate $P2X_2/P2X_3$ receptors (McQueen et al., 1998; Prasad et al., 2001), and ATP's metabolite, adenosine, can also increase CSN activity.

The experiments described in this work were performed in whole CBs, which have a complex fine structure with glomus (type I) cells, sustentacular (type II) cells, capillaries, carotid sinus nerve sensitive fibres, autonomic nerve endings, etc. (Verna, 1997). It is not possible to assess the contribution of each of these structures to the production and release of adenosine during moderate hypoxia. The cellular origin of ATP (and its metabolite, adenosine) could be different than that of adenosine. ATP is co-stored with catecholamines in glomus cells (Eyzaguirre and Zapata, 1984) and co-released with ACh (Zhang et al., 2000), which is not synthesized in glomus cells but may be made in, and released from, nerve fibres (Gauda, 2002).

In summary, evidence for two metabolic sources of extracellular adenosine, catabolism of ATP by ecto-5'-nucleotidase, and adenosine transport by ENT, were obtained for the rat carotid body. Hypoxia can trigger the release of adenosine by itself through an ENT sensitive to NBTI. Arterial tissue and SCG were less sensitive to hypoxia in terms of adenosine release. We postulate that specific CB cells (e.g. type I, type II or CSN endings) may have a low threshold for the release of adenosine in response to hypoxia, and this may contribute to the regulation of CB chemoreceptor sensitivity.

4. CHAPTER 2 – EFFECT OF ACTIVATION OF NICOTINIC ACH RECEPTORS WITH α4 SUBUNITS ON ADENOSINE RELEASE AT THE RAT CAROTID BODY

4.1 Introduction and aim

As previously described in section 1.5.4.2. of the general introduction, ACh nicotinic receptors are present at the CB in glomus cells (Dinger et al., 1981a; Dasso et al., 1997; Obeso et al., 1997) and in nerve fibres (Shirahata et al., 1998) and it is agreed that nicotinic ACh receptors in glomus cells act as modulators, increasing $[Ca^{2+}]_i$ (Dasso et al., 1997) and inducing the release of neurotransmitters at the CB, such as dopamine (Obeso et al., 1997a). Since ATP and adenosine are released from rat CB in response to hypoxia (Buttigieg and Nurse, 2004; Chapter 1 of this thesis) and have been proposed as excitatory neurotransmitters at the CB in animal models, we postulate that the excitatory effects of ACh at the CB could involve the release of other excitatory neurotransmitters. In this chapter we investigate if the activation of nicotinic ACh receptors can stimulate adenosine release at the CB. To test this hypothesis whole CBs from rats were used and a pharmacological functional characterisation of the nicotinic receptors was performed. Since adenosine at the CB can originate both from release through nucleoside transporters and extracellular catabolism of ATP, the contribution of extracellular ATP degradation to adenosine production induced by nicotinic stimulation was also studied.

4.2. Material and methods

4.2.1. Animals and surgical procedures

Experiments were performed in Wistar adult rats (250-350 g) from the Faculty of Medical Sciences animal house, kept at a constant temperature (21ºC) and a regular light (08.00-20.00h) and dark (20.00-08.00h) cycle, with food and water *ad libitum*. All the surgical procedures were performed as previously described in section 3.2.1. After 30 min in hyperoxia (95% O_2 + 5% CO_2) (recovery period) at 37º C the CBs were incubated for 10 min in Tyrode solution equilibrated with 20% O_2 + 5% CO_2 (normoxia) or 10% O_2 + 5% CO_2

(hypoxia) and in the presence of 2.5 μM of EHNA, an inhibitor of adenosine deaminase, to avoid adenosine degradation.

4.2.2. Effect of ACh nicotinic receptor agonists on adenosine released from carotid body

The effect of ACh nicotinic receptor agonists was assessed in normoxic conditions (20% O_2 + 5% CO_2). Nicotinic receptor agonists used were ACh (30μM), cytisine (Cyt) (0.01-10 μM), dimethylphenylpiperazinium (DMPP, 0.1 – 300 μM) and nicotine (0.1 – 100 nM). In each experiment only one agonist concentration was tested and added to the incubation medium that contained 2.5 μM of EHNA, in the last 3 minutes of the incubation period (10 min) to avoid nicotinic ACh receptor desensitisation. In the experiments with ACh, an inhibitor of AChE, physostigmine was added to the incubation medium and two different concentrations (30 and 300 μM) were tested.

After the incubation period, the CBs were removed from the medium and the nucleotides were extracted from the incubation medium.

4.2.3. Effect of ACh nicotinic receptor antagonists on adenosine released from carotid body

The effect of ACh nicotinic receptor antagonists was assessed in hypoxic conditions (10% O_2 + 5% CO_2). Endogenous ACh released by the CB in response to its physiological stimulus, hypoxia, may activate nicotinic ACh receptors different from those stimulated by exogenously applied agonist. However, only characterisation of nicotinic ACh receptors that are involved in the release of adenosine under physiological conditions was considered here. The dose-response curves for the effects of d-tubocurarine (0.001 – 200 μM), mecamylamine (0.001 – 100 μM), di-hydro-β-erythroidine (DHβE, 0.001 – 100 μM) and α-bungarotoxin (0.001 - 10 μM) on the release of adenosine were performed in CBs stimulated by 10 min of hypoxia. The ACh nicotinic receptor antagonists were included in the medium at the beginning of the recovery period in hyperoxia, in order to try to obtain an adequate diffusion of the drug and the block of the nicotinic receptors. After the incubation period in hypoxia,

the CBs were removed and the nucleotides were extracted from the incubation medium.

4.2.4. Pharmacological demonstration of the involvement of neuronal nicotinic acetylcholine receptors

To demonstrate the involvement of neuronal nicotinic receptors, the effect of the selective nicotinic receptor antagonist, DHβE, on the release of adenosine from CBs was assessed by incubating CBs with a maximal dose (100 μM) of DHβE in normoxia (20% O_2 + 5% CO_2) for 10 min in the presence of nicotine (0.1 - 1 μM). After the incubation period, the CBs were removed and the nucleotides were extracted from the incubation medium.

4.2.5. Effect of extracellular ATP catabolism inhibitor on the release of adenosine evoked by nicotine

The effect of the AOPCP on the release of adenosine from CBs was assessed by incubating CBs with 100 μM of AOPCP in normoxia (20% O_2 + 5% CO_2) for 10 min in the presence of 100 nM of nicotine. After the incubation period, the CBs were removed and the nucleotides were extracted from the incubation medium.

4.2.6. Nucleotide extraction and HPLC analysis

Nucleotides were extracted from the medium as previously described in section 3.2.6. Aliquots of neutralised supernatant were collected and kept at –20ºC until analysis by HPLC. The samples were analysed in triplicate by reverse-phase HPLC with UV detection at 254 nm as described in section 3.2.7.

4.2.7. Drugs and chemicals

ACh, adenosine, AOPCP, α-bungarotoxin, cytisine, DHβE, DMPP, EHNA, mecamylamine, nicotine, d-tubocurarine, physostigmine, and Sigmacote

were all from Sigma. Cytisine and physostigmine were made up in a 5 and 30 mM stock solution in DMSO and ethanol, respectively. All stock solutions were stored as frozen aliquots at –20ºC. Dilutions of stock solutions were made in Tyrode solution in accordance with the drug concentration used.

4.2.8. Data analysis

The amount of adenosine present in the incubation medium was expressed in pmol/CB. Data were evaluated using Graph Pad Prism Software, version 4 and were presented as mean ± SEM. The significance of the differences between the means was calculated by unpaired Student's *t* test. *P* values of 0.05 or less were considered as representing significant differences.

4.3. Results

4.3.1. Demonstration of the involvement of neuronal nicotinic acetylcholine receptors and characterisation of nicotinic receptors that modulate the release of adenosine from the rat carotid body

The basal levels of adenosine released from rat CBs during 10 min of normoxia were 113.3 ± 11.77 pmol/CB (n = 5). The effect of ACh on the amount of adenosine released from the intact rat CB in normoxia is shown in Figure 22. ACh (30 μM) plus physostigmine (300 μM) for 10 min caused a slight but statistically insignificant increase to 122.75 ± 15.33 pmol/CB (n = 5), which corresponds to an increase of 8.34% in adenosine concentrations (Figure 22). Since it has been previously shown that this AChE-inhibiting drug, physostigmine, in high concentrations (>100 μM) is a competitive ligand of ACh on α4-containing nicotinic ACh receptors (Zwart et al., 2000) a lower concentration (30 μM) was tested.

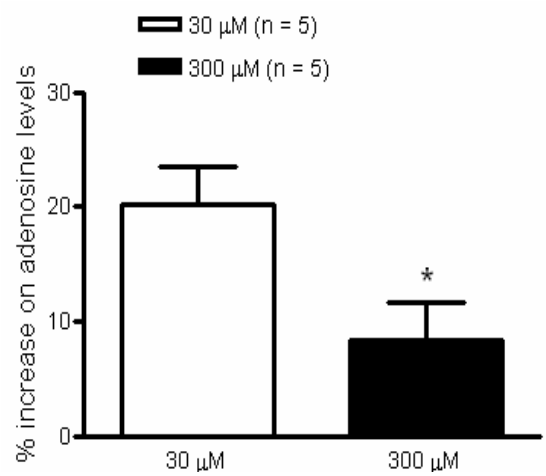


Figure 22 Effect of acetylcholine (ACh, 30 µM) on adenosine concentrations released from rat carotid bodies (CB) in the presence of distinct concentrations of physostigmine (30 and 300 µM, n = 5). All the experiments were performed in normoxia and in the presence of EHNA (2.5 µM). 0% increases correspond to adenosine concentrations of 113.30 ± 11.77 pmol/CB (n = 5) in normoxia.* *P<0.05*; unpaired *Student's t* test corresponding to the differences in the effects of distinct doses of physostigmine. Bars represent means ± SEM.

As can be observed in Figure 22, ACh (30 µM) induced a statistically significant increase of 20.2 ± 3.3 % (n = 5) in the concentration of adenosine released in the presence of 30 µM of physostigmine. To avoid the interaction of AChE inhibitors with nicotinic receptors, more stable agonists of nicotinic ACh receptors, namely cytisine, dimethylphenylpiperazinium (DMPP) and nicotine were tested.

Cytisine (0.01 – 10 µM), DMPP (0.1 – 300 µM) and nicotine (0.1 – 100 nM) increased the release of adenosine in the CBs in normoxia in a concentration-dependent manner (Figure 23).

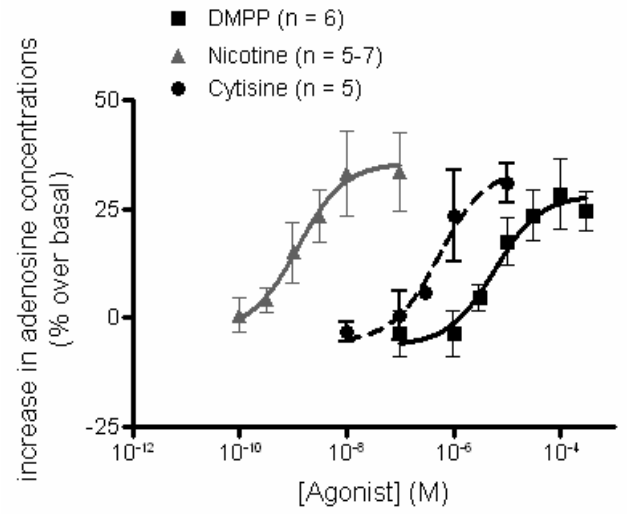


Figure 23 Dose-response curves for the effects of nicotinic ACh receptor agonists, cytisine (n = 5), dimethylphenylpiperazinium (DMPP, n = 6) and nicotine (n = 5 - 7) on adenosine concentrations released from rat carotid bodies in normoxia. 0% increases correspond to adenosine concentrations of 113.3 ± 11.77 pmol/CB (n = 5) in normoxia. Vertical bars represent means ± SEM.

Maximal increases (Emax) in the release of adenosine occurred with concentrations of 10 µM cytisine, 100 µM DMPP and 100 nM nicotine. The EC_{50}

(drug concentration that produced 50% of maximal effect), and Emax, obtained from dose-response curves of nicotinic agonists, are represented in Table 4.

Table 4 Efficacy and potency of nicotinic ACh receptor agonists in stimulating adenosine release at the carotid body.

Agonist	Emax (% effect)	EC_{50}
Cytisine (n = 5)	34.15 ± 6.8	0.54 μM
DMPP (n = 6)	28.27 ± 4.1	5.8 μM
Nicotine (n = 5/7)	35.63 ± 6.0	1.2 nM

DMPP: dimethylphenylpiperazinium. Emax: maximal increase (%) in the release of adenosine (mean ± SEM). EC50: drug concentration that produced 50% of maximal effect.

The effect of nicotinic ACh antagonists on the amount of adenosine released from rat CBs was assessed during physiological stimulation of chemoreceptor cells with hypoxia (10% O_2). Dose-response curves for the effect of three nicotinic ACh receptor antagonists, d-tubocurarine, di-hydro-β-erythroidine (DHβE) and α-bungarotoxin as well for the effect of the allosteric inhibitor of nicotinic ACh receptor, mecamylamine, on adenosine release from rat CBs elicited by hypoxia are shown in Figure 24. As had been previously shown (Chapter 1, Section 3 of the current work), hypoxia by itself increased the release of adenosine in the CB to 152.1 ± 5.2 pmol/CB (0% effect, n = 5). D-tubocurarine (0.001 – 100 μM), mecamylamine (0.001 – 100 μM), DHβE (0.001 – 100 μM) and α-bungarotoxin (0.001- 10 μM) caused a concentration-dependent decrease in adenosine concentrations released from the rat CBs in response to hypoxia, with the effect of α-bungarotoxin being very small (Emax= 22.2 ± 3.4%, Figure 24). The maximal inhibitory effect (Emax) on adenosine release was obtained with 100 μM of DHβE (Emax = 70.39 ± 2.4%, Figure 24).

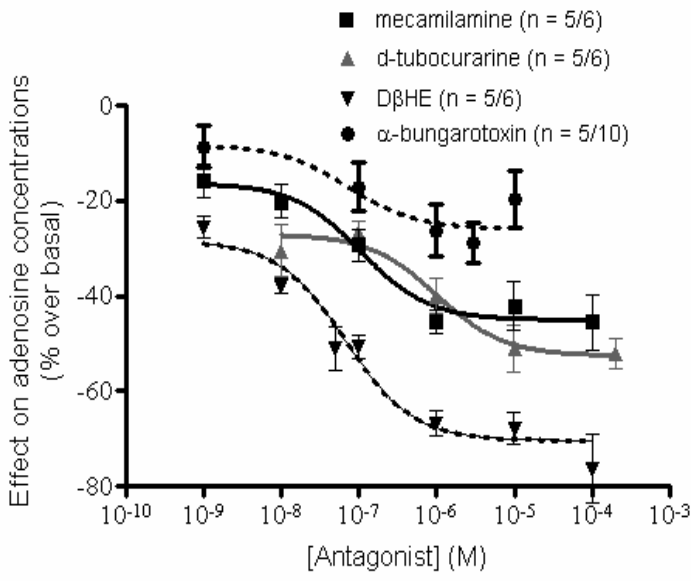


Figure 24 Effects of nicotinic ACh receptor antagonists, α-bungarotoxin, d-tubocurarine, and di-hydro-β-erythroidine (DHβE) and of the allosteric inhibitor, mecamylamine, on the release of adenosine from rat CBs stimulated by hypoxia (10% O_2). 0% effect corresponds to adenosine concentrations of 152.1 ± 5.2 pmol/CB (n = 5) in response to hypoxia. Data are means ± SEM (n = 5-6).

IC_{50} (drug concentrations that caused 50% of maximal inhibition) and Emax values obtained from the dose-response curves for the nicotinic ACh receptor antagonists are represented in Table 5. These results showed a rank order of potency with DHβE > mecamylamine > d-tubocurarine > α-bungarotoxin.

Table 5 Efficacy and potency of nicotinic ACh antagonists in inhibiting the release of adenosine in CBs stimulated by hypoxia.

Antagonist	Emax (% effect)	IC_{50} (μM)
α - bungarotoxin	-22.2 ± 3.4	_____
d-tubocurarine	-52.56 ± 2.7	1.06
Mecamylamine	-45.03 ± 2.8	0.10
DHβE	-70.39 ± 2.4	0.067

DHβE: di-hydro-β-erythroidine. Emax: maximal % of inhibition (mean ± SEM). IC50: drug concentrations that caused 50% of the maximal inhibition.

To demonstrate the involvement of neuronal nicotinic receptors, the effect of the selective nicotinic receptor antagonist, DHβE on the release of adenosine from CBs evoked by nicotine was studied. It was observed that DHβE inhibits the effect of nicotine on adenosine release from CB, and this inhibition was complete when the concentration of nicotine (100 nM) that

produces the maximal effect on adenosine release from CB was used. To observe increases in adenosine release from CB evoked by nicotine in the presence DHβE, it was necessary to increase the dose of nicotine applied (Figure 25). Looking at Figure 25, it seems that the inhibition by DHβE moves the dose-response curve for the release of adenosine evoked by nicotine to the right.

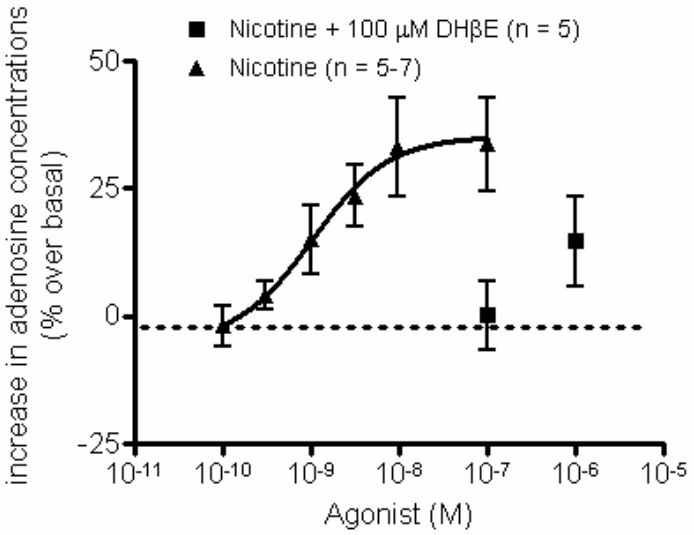


Figure 25 Effect of the selective nicotinic receptor antagonist, di-hydro-β-erythroidine (DHβE; 100 µM) on the release of adenosine evoked by nicotine (0.1 - 1 µM) during normoxia (20% O_2 + 5% CO_2). Values represent means ± SEM (n = 5).

4.3.2. Effect of inhibition of ATP catabolism on the release of adenosine evoked by nicotine on carotid body

In order to investigate whether nicotinic ACh receptor activation induced the release of adenosine *per se* or induced the release of ATP (this being the nucleotide that is further metabolised into adenosine) experiments in CBs stimulated by nicotine (Nic) in a concentration (100 nM) that induced a maximal release of adenosine were performed in the presence of the inhibitor of ecto-5'-nucleotidase, AOPCP. AOPCP (100 µM), used in a concentration 10 times higher than that which inhibits 90% of AMP hydrolysis by ecto-5'-nucleotidases (Meghji and Burnstock, 1995), reduced the release of adenosine by 72% ($P < 0.001$) in the CBs stimulated by nicotine (Figure 26).

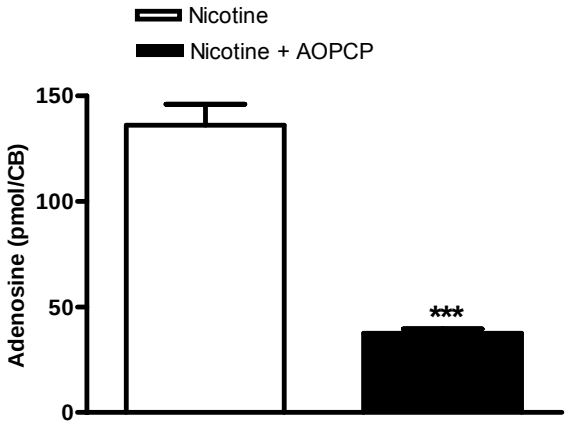


Figure 26 Effect of 100 μM of α,β-methylene ADP (AOPCP) on the release of adenosine in CBs stimulated by 100 nM of nicotine in normoxia. *** *P*<0.001; unpaired *Student's t-test* corresponding to the differences observed in the presence or absence of AOPCP. Data are means ± SEM (n = 5).

4.4. Discussion

Activation of ACh nicotinic receptors at the CB in normoxia stimulated the release of adenosine that apparently comes mainly from extracellular degradation of ATP. The increase in the amount of adenosine at the CB induced by hypoxia was partially antagonized by ACh nicotinic receptor antagonists. The rank order of potency for the effect of nicotinic agonists and antagonists on the release of adenosine was, respectively: nicotine > cytisine > DMPP and DHβE > mecamylamine > d-tubocurarine > α-bungarotoxin.

The characterisation of nicotinic receptor subunits in the CB of the rat has never been performed. However, in the cat, immunohistochemical and RT-PCR techniques have reported the presence of α_3, α_4 and β_2 subunits in glomus cells (for a review see Higashi et al., 2003) and α_7 subunits in nerve fibres surrounding the glomus cells (Shirahata et al., 1998). In this work we have described the involvement of neuronal nicotine ACh receptors in stimulating the release of adenosine from CBs and characterized the nicotinic ACh receptor involved. The effect of the endogenous agonist, ACh, was mimicked by nicotine (nicotinic ACh receptor agonist), used in a concentration that induces a maximal release of adenosine, this effect being antagonized by DHβE (a selective nicotinic ACh receptor antagonist), demonstrating the involvement of a neuronal nicotinic ACh receptor in the induction of adenosine release from rat CBs. The rank order of potency obtained for the nicotinic ACh receptor agonists studied is compatible with nicotinic receptors that contain subunits $\alpha_4\beta_2$ (Nic > cyt > DMPP) (Alexander et al., 2004). The presence of nicotinic receptors containing

α_2, α_3 subunits can be excluded, since receptors containing α_2 subunits have a comparable affinity for cytisine and nicotine and lower affinity for DMPP, and receptors containing α_3 subunits have a higher affinity for DMPP than for cytisine (Alexander et al., 2004; Jensen et al., 2005). Concerning the effect of the nicotinic receptor antagonists, a rank order of potency of DHβE > d-tubocurarine > α-bungarotoxin was observed for the inhibition of the release of adenosine from CB during hypoxia. Mecamylamine was excluded from this pattern since it is a non-selective allosteric inhibitor of nicotinic receptors with major affinity for nicotinic receptors containing α_3 subunits (Jensen et al., 2005). As was found for the agonists, the pattern of inhibition is compatible with the presence of nicotinic receptors with $\alpha_4\beta_2$ subunits (Jensen et al., 2005). The absence of consistent effects of α-bungarotoxin inhibiting the release of adenosine from CB during hypoxia excludes the presence of nicotinic receptors with α_7, α_8, α_9 and α_{10} subunits. These findings agree with the description of both α_4 and β_2 subunit transcription in total CB RNA in mice (Cohen et al., 2002) and in glomus cells (Higashi et al., 2003) and with nicotinic ACh receptor $\alpha_4\beta_2$ subunits modulating the release of neurotransmitters in the human central nervous system (Champtiaux et al., 2003).

The experiments herein described were performed in whole CBs and do not provide evidence concerning the cell origin of adenosine and/or nicotinic receptor localization, but previous evidence suggests that they are present in glomus cells. Nicotinic ACh receptors with α_4 and β_2 subunits were described in glomus cells (Higashi et al., 2003) and could act as modulators, increasing $[Ca^{2+}]_i$ (Dasso et al., 1997) and inducing the release of several neurotransmitters at the CB, like DA (Obeso et al., 1997a) and ATP. Adenosine can be produced by different cells at the CB in response to nicotinic activation. However, in response to acute moderate hypoxia (10% O_2) significant increases in the amount of adenosine were found at the CB but not in other structures - arterial tissue or superior cervical ganglions - present at the CB and devoid of chemosensitive properties (Chapter 1, Section 3), suggesting that adenosine originates from glomus chemosensitive cells.

The maximal increase in the release of adenosine (36%) induced by nicotinic agonists was similar to that (35%) caused by acute moderate hypoxia in the same conditions, but nicotinic antagonists do not completely abolish the

stimulatory effect of hypoxia on adenosine production. This evidence together with the differences between the Emax found for d-tubocurarine and for DHβE could indicate that other, different nicotinic receptor subunits could be involved. For example, there might be α_4 and β_2 subunits associated with α_5 subunits, as is described in central nervous system in cells that release dopamine (Champtiaux et al., 2003). Another interpretation is that hypoxia can trigger adenosine production through two different mechanisms: one independent and another dependent on nicotinic receptor activation. Further investigation and more specific drug tools are needed to clarify this point but promising data can be advanced based on adenosine concentrations obtained during hypoxia and nicotinic activation when extracellular catabolism of ATP was inhibited with AOPCP. Inhibition of ecto-5'-nucleotidases with AOPCP reduced the amount of nicotine-induced extracellular adenosine by 72% (present work), but in the same experimental conditions caused a reduction of only 44% in adenosine production in response to hypoxia (Chapter 1, Section 3). Adenosine is a common pathway of the CB response to both hypoxia and nicotinic activation, but while moderate hypoxia stimulates both intracellular production of adenosine and the release of ATP (Chapter 1, Section 3), nicotinic activation preferentially induced the release of ATP. The proportion of ATP molecules that can stimulate P_2 receptors before being deactivated by ecto-nucleotidases is not known, but the classical studies performed by McQueen and Ribeiro (1983; 1986) strongly support the theory that part of the excitatory effects of ATP on carotid sinus nerve is mediated by its metabolite adenosine.

The reduction in adenosine release induced by ACh that is caused by high concentrations of physostigmine, and previous indications that physostigmine is a competitive ligand of ACh on nicotinic receptors that contain α_4 subunits (Zwart et al., 2000), together support the characterisation of the $\alpha_4\beta_2$ nicotinic receptor as containing subunits that stimulate adenosine release at the CB.

Interactions between adenosine and ACh in the central nervous system and peripheral nerve endings have been described (for a review see e.g. Ribeiro et al., 1996). It is generally accepted that adenosine acts as a modulator of ACh release: selective activation of A_1 and A_2 adenosine receptor subtypes causes respectively inhibitory and excitatory effects on ACh release (Ribeiro et

al., 1996). In whole cat carotid bodies it was recently shown that exogenous adenosine stimulates the release of ACh in hypoxic (4% O_2) or hyperoxic (40% O_2) conditions (Fitzgerald et al., 2004). This work does not provide evidence on the type of adenosine receptors, mechanisms or cell types involved (Fitzgerald et al., 2004), but is consistent with the hypothesis that the effects of both excitatory transmitters – adenosine/ATP and ACh - act by synergistic mechanisms at the CB. The characterisation of A_2 receptors at the CB, localized in several structures (glomus cells, vessels, nerve endings) was extensively done *in vitro* (Gauda et al., 2000; Kobayashi et al., 2000; Monteiro et al., 1996) and *in vivo* (McQueen and Ribeiro, 1986; Ribeiro and Monteiro, 1991). It was recently demonstrated that, at the CB, ACh apparently comes from nerve endings instead of glomus cells (Gauda et al., 2004a), which is compatible with its action on nicotinic receptors with α4 subunits in glomus cells, stimulating the release of ATP/adenosine. In contrast to the well-known modulatory role for adenosine in cholinergic transmission, we are not aware of the information relating to the consequences of nicotinic receptor activation on adenosine release in other preparations. However, in chromaffin cells or PC12 cells, preparations with great similarities to the carotid bodies, it is known that nicotinic activation induces the release of ATP (Rojas et al., 1985) and catecholamines (Courtney et al., 1991; Nagayama et al., 1999).

Although the involvement of nicotinic ACh receptors in hypoxia signalling is not a novel concept, here we demonstrate for the first time that α_4 subunits present at the CB are functionally active during hypoxia and that activation of these receptors by ACh induce the production of adenosine originating mainly from extracellular catabolism of ATP through the action of ecto-5'-ectonucleotidases. These findings suggest that the excitatory effects caused by ACh in chemosensory activity include indirect activation of purinoreceptors by adenosine and ATP, which strongly supports the hypothesis that ATP/adenosine are important excitatory mediators in chemotransduction.

5. CHAPTER 3 – ACUTE EFFECTS OF CAFFEINE ON RAT CAROTID BODY CHEMORECEPTOR FUNCTION

5.1. Introduction and aim

As discussed in the general introduction, caffeine has been described as having both excitatory and inhibitory effects on ventilation. It is also known that adenosine in peripheral chemoreceptors is a stimulating agent (see e.g.: Monteiro and Ribeiro, 1987, 1989; Runold et al., 1990). Caffeine at low concentrations, those obtained with common coffee intake, is an antagonist of adenosine receptors. The aim of this chapter is to investigate the effects of acute administration of caffeine on peripheral control of breathing and the identity of adenosine receptors involved in the effects of adenosine and caffeine on the chemoreceptors of the rat CB. For that we have used a combination of neurochemical and pharmacological techniques to assess the effect of caffeine and specific adenosine antagonists on CSN chemosensory activity and on the release of CA from rat CB. We also used immunocytochemical techniques to locate specific adenosine receptors involved in caffeine effects on chemoreceptor cells of rat CB.

5.2. Material and methods

5.2.1. Animals and surgical procedures

Experiments were performed in Wistar adult rats of both sexes (250-350 g) obtained from animal house of the Faculty of Medicine of the University of Valladolid. The Institutional Committee of the University of Valladolid for Animal Care and Use approved the protocols. Rats were anaesthetized with sodium pentobarbital (60 mg/kg i.p.), tracheostomized and the carotid arteries were dissected past the carotid bifurcation.

For the release of CA experiments (4-12 CBs/experiment) and for cell dissociation (4 CB/experiment), the CBs were cleaned free of CSN and nearby connective tissue following the procedures previously described (Vicario et al., 2000a). For the recording of CSN activity, the preparation CB-CSN was identified under a dissecting microscope and a block of tissue, including the carotid bifurcation and the glossopharyngeal nerve, was removed and placed in

a Lucite chamber in ice-cold 100% O_2-equilibrated Tyrode (in mM: NaCl 140; KCl 5; $CaCl_2$ 2; $MgCl_2$ 1.1; HEPES 10; glucose 5.5, pH 7.40) for further dissection of tissue surrounding CB and CSN. The preparation CB-CSN was digested for 3-5 min with collagenase type I (1mg/ml) to loosen the perineurium (Rigual et al. 2002). The CB-CSN preparation was maintained in ice-cold 100% O_2-equilibrated Tyrode until it was transferred to the recording chamber. In all instances animals were killed by intracardiac overdoses of sodium pentobarbital until the beating of their hearts ceased.

5.2.2. Labelling of catecholamine stores: release of ^{3}H-CA

To monitor the release of CA we used a radioisotopic method as described by Fidone et al. (1982) and Vicario et al. (2000a). The stores of CA in the CB were labelled by incubating the organs in a solution containing tyrosine, the natural precursor of CA, labelled with tritium (3,5-^{3}H-tyrosine, Amersham). 4 to 12 CBs were placed in small glass vials containing 0.5 ml of Tyrode solution and placed in a shaker bath at 37 ºC. The concentration of ^{3}H-tyrosine was 30 µM and its specific activity was 45 Ci/mmol. In addition, the incubating solution contained 100 µM 6-methyl-tetrahydropterine and 1 mM ascorbic acid, cofactors for tyrosine hydroxylase and dopamine-β-hydroxylase, respectively (Fidone and Gonzalez, 1982). Incubation lasted 2h. After the labelling period, individual CBs were transferred to vials containing 4 ml of precursor-free Tyrode-bicarbonate solution (composition as above except for the substitution of 24 mM of NaCl for 24 mM of $NaHCO_3$). Solutions were continuously bubbled with 20%O_2/5% CO_2/75% N_2 saturated with water vapour, except when hypoxic stimuli were applied. The solutions of the initial incubation periods (3x20min) were discarded to washout the precursor and the readily releasable pool of labelled CA (Almaraz et al., 1986), and were thereafter renewed at fixed times (every 2 to 10 min; see Results) and collected for subsequent analysis of their ^{3}H-CA content. Specific protocols for stimulus and drug applications are provided in the Results. Stimuli included hypoxias of two intensities (10 and 5% O_2-equilibrated solutions) and high external K^+ (30 and 50 mM). In addition to caffeine (0.01-10 mM) we used: 2-p-(2-carboxyethyl)phenethyl-amino-5'-N-ethyl-carboxamido-adenosine hydrochloride (CGS 21680, an A_{2A} agonist; 1µM), 8-cycle-1,3-

dipropylxanthine (DPCPX, an A_1 and A_{2B} antagonist; 0.15 and 1.5 μM), 2-hexynyl-5'-ethylcarboxamidoadenosine (HE-NECA, an A_{2A} agonist; 2 μM), 8-[4-[((4-cyanophenyl)carbamoylmethyl)oxy]phenyl]-1,3-di(n-propyl)-xanthine (MRS 1754, an A_{2B} antagonist; 2, 20 and 200 nM), 5'-(N-ethylcarboxamido)adenosine (NECA, an adenosine A_2 agonist; 1 μM) and SCH 58621 (an A_{2A} antagonist; 20 and 200 nM).

Collected solutions were acidified with glacial acetic acid to pH = 3 and maintained at 4º C until analysis to prevent ^{3}H-CA degradation. ^{3}H-CA present in the incubating solutions was analysed by adsorption onto alumina at alkaline pH (pH = 8.6) and bulk elution with hydrochloric acid (1 N) and quantified by scintillation counting. CB were homogenised (glass to glass; 4 ºC) in 200 μl of perchloric acid 0.6 M and centrifuged for 10 min (4º C; 12000 g), in a microfuge (Beckmann, Madrid). Supernatants were analysed for their ^{3}H-catechol content as described above for the incubating solutions. Previous HPLC analyses have shown that over 90% of the ^{3}H-catechols present in the alumina eluate corresponds to ^{3}H-DA and its catabolite ^{3}H-DOPAC (Vicario et al., 2000a).

5.2.3. Recording of carotid sinus nerve activity

The CB-CSN preparation (Figure 27B) was transferred to a recording chamber mounted on a dissection microscope (Nikon Corporation, Tokyo, Japan) and superfused (37 ºC) with bicarbonate/CO_2 buffered saline (in mM: NaCl 120; $NaHCO_3$ 24; KCl 3; $CaCl_2$ 2; $MgCl_2$ 1.1; glucose 10, pH 7.40). Recordings of either a single or a few fibres of CSN were made using a suction electrode. The pipette potential was amplified (Neurolog Digimiter, Hertfordshire, England), displayed on an oscilloscope and stored on computer (200 Hz acquisition rate, Axonscope, Axon Instruments, USA). Chemoreceptor activity was identified (spontaneous generation of action potentials at irregular intervals) and confirmed by its increase in response to hypoxia (normoxia: 20% O_2 + 5% O_2 + 75% N_2; hypoxia: either 0% or 5% O_2 + 5% CO_2 + balanced N_2). CSN activity was digitalized, summed every second and converted into a voltage proportional to the sum. The effects of caffeine (1 nM to 10 mM), SCH 58621 (20-200nM) and ZM 241385 (300nM) on the CSN activity were studied

while perfusing the preparations with normoxic (20% O_2-equilibrated) and hypoxic (5% O_2-equilibrated) solutions.

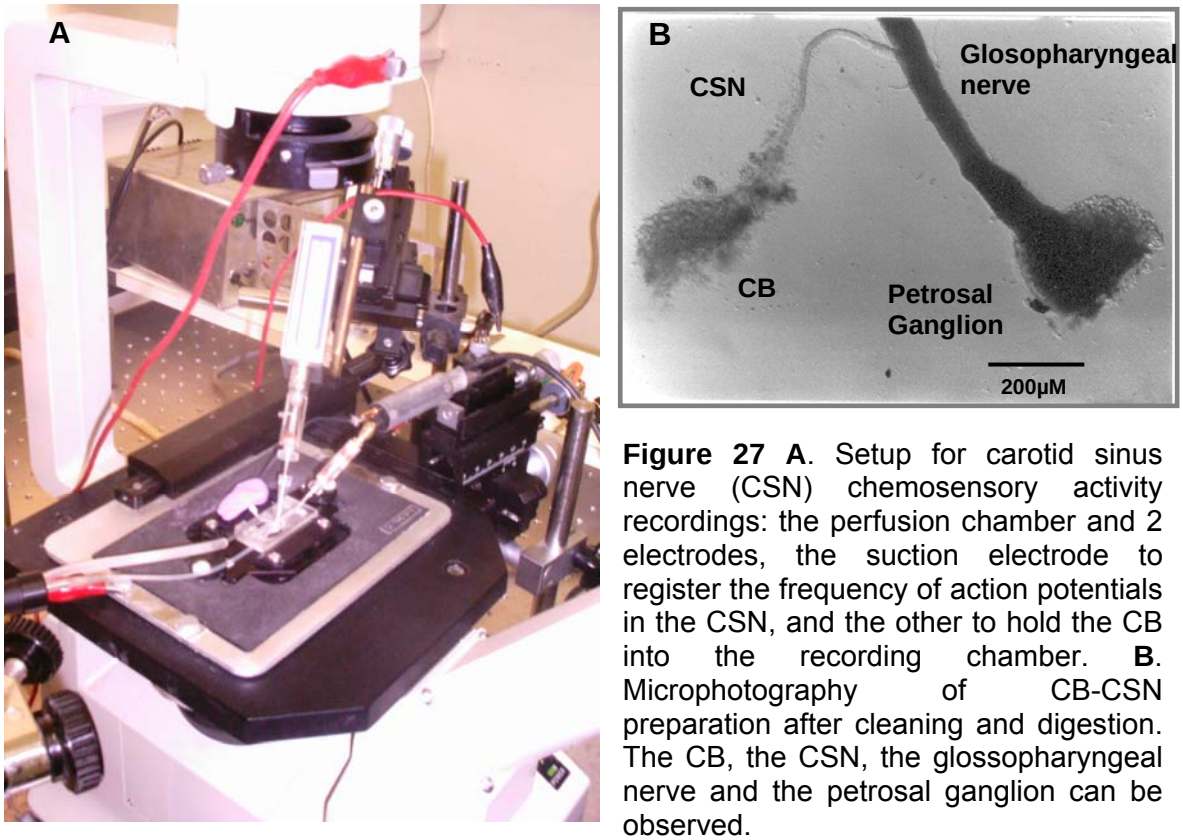


Figure 27 A. Setup for carotid sinus nerve (CSN) chemosensory activity recordings: the perfusion chamber and 2 electrodes, the suction electrode to register the frequency of action potentials in the CSN, and the other to hold the CB into the recording chamber. **B**. Microphotography of CB-CSN preparation after cleaning and digestion. The CB, the CSN, the glossopharyngeal nerve and the petrosal ganglion can be observed.

5.2.4. Carotid body cell dissociation and culture, and immunocytochemistry

CBs were enzymatically digested in 5 ml conical tubes in two consecutive steps: incubation with solution 1 containing collagenase 2.5 mg/mL (EC 3.4.24.3) and albumin 6mg/mL (15 min, 35º C) and incubation with solution 2 containing trypsin 1 mg/mL (EC 3.4.21.4) and albumin 6 mg/mL (20 min, 37 ºC); both solutions were prepared in 0 Ca^{2+} and 0 Mg^{2+} Tyrode. After the second incubation the organs were allowed to sediment, the enzymatic solution was removed, 1 ml of Dulbecco's modified Eagle's medium (DMEM) at 4 ºC was added and the CBs were mechanically dispersed with a polished Pasteur pipette at room temperature. The samples were centrifuged for 8 min at 2000 rpm and the supernatants were eliminated. The pellet was re-suspended in 50 µL of DMEM medium and plated on poly-L-lysine coated glass coverslips in

drops of about 15-20 μl. After 30 min incubation (37º C, 5% CO_2), when the cells were attached, 500 μL of DMEM medium was added to the wells containing the coverslips. CB cells were kept in the incubator for 8 to 24 hours until used.

For immunocytochemistry of A_{2B} adenosine receptors and its co-localization with TH in chemoreceptor cells of CB, specific antibodies to these two proteins were used: a mouse anti-TH, monoclonal antibody against TH (Abcam, Cambridge, UK) and rabbit anti-A_{2B}, polyclonal antibody against Adenosine A_{2B} receptors (Chemicon International, Temecula, CA, USA). Coverslips were washed with phosphate buffer solution (PBS; 2 x 5 min) and fixed with 4% paraformaldehyde in PBS at room temperature (15 min) and finally the fixative was washed away with PBS at room temperature (3 x 5 min). Cells were then exposed to a blocking-permeabilising solution containing 2% of goat serum and 0.1% of Triton X-100 in PBS for 20 min at room temperature, followed by an overnight incubation at 4º C in a humidified chamber with the following cocktail of primary antibody: mouse anti-TH (1:1000) and rabbit anti-A_{2B} (1:50). The primary antibody was diluted in PBS containing 0.6 M NaCl, 0.1% Triton X-100 and 2% of goat serum. Thereafter, coverslips were washed with PBS (3 x 10 min) and incubated for 1 hour and 30 min at room temperature with secondary antibodies. These consisted in goat anti-mouse IgG conjugated to FITC (1:1000, Sigma ImmunoChemicals, Madrid, Spain) and goat anti-rabbit conjugated to Alexa 594 (1:2000, Molecular Probes, Oregon, USA). Secondary antibodies were diluted in PBS containing 0.6 M NaCl, 0.1% Triton X-100 and 2% of goat serum. After incubation with secondary antibodies the cells were washed with PBS (3 x 10 min) and with distilled water and covered with an aqueous mounting medium Vectashield (Vector Laboratories, Burlingame, CA, USA). In control experiments, omission of the primary antibody resulted in the abolition of staining in all cases.

5.2.5. Drugs and Chemicals

6-methyl-tetrahydropterine, ascorbic acid, bovine serum albumin, caffeine, collagenase (EC 3.4.24.3), CGS 21680, DPCPX, HE-NECA, MRS 1754, NECA, trypsin (EC 3.4.21.4), were all obtained from Sigma (Sigma-

Aldrich, Spain). Collagenase type I (292 U/mg) was obtained from Worthington (Lakewood, NJ, USA). ZM 241385 was obtained from AstraZeneca (UK). ^{3}H-tyrosine was obtained from Amersham (Madrid, Spain). SCH 58621 was kindly offered by Dr. L. V. Lopes (School of Medicine, University of Lisbon). HE-NECA, NECA, SCH 58621 and ZM 241385 were prepared as 5 mM stock solutions in DMSO; the final concentration of DMSO was always below 1/500 which, by itself, lacks effects on our preparations. The rest of the chemicals used were from Merck, Darmstadt, Germany.

5.2.6. Data analysis

Data were evaluated using Graph Pad Prism Software, version 4 and were presented as mean ± SEM. Dose-response curves were fitted to Michaelis-Menten curves in order to obtain the 0% effect of the drug in each situation and the IC50 and Emax values obtained directly from the Michaelis-Menten equations. The significance of the differences between the means was calculated by unpaired Student's *t* test and by One and Two-Way Analysis of Variance (ANOVA) with Dunnett's and Bonferroni multiple comparison tests, respectively. *P* values of 0.05 or less were considered as representing significant differences.

5.3. Results

5.3.1. Effect of caffeine on the basal release of ^{3}H-CA

After the initial washout period (see Materials and Methods) the basal-normoxic (20% O_2 equilibrated solutions; PO_2 ≈130 mmHg) release of ^{3}H-CA follows a monotonic slow decay (Almaraz et al., 1986). Figure 28A shows that the addition of caffeine (10 mM) to the incubating solutions produced a marked inhibition of this basal release that recovered slowly after the elimination of the drug, the inhibition being nearly identical in Ca^{2+}-free solutions.

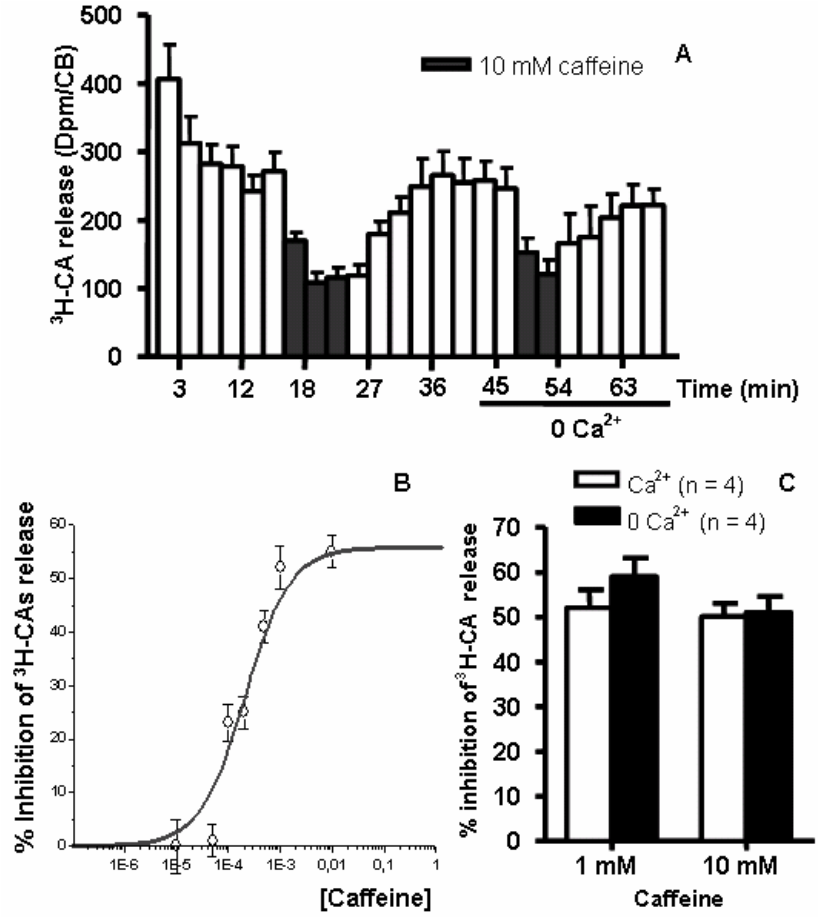


Figure 28 Effect of caffeine on the basal (normoxic) release of catecholamines (CA) from the intact rat carotid body *in vitro* and its Ca^{2+} dependency. **A.** Mean (n=4) time course of the basal release of catecholamines. Each column represents the ^{3}H-CA (dpm) present in the incubating solution collected every 3 min. The incubating solutions correspondent to the fraction marked in grey contained 10 mM caffeine. Ca^{2+}-free solutions were used during the marked periods. **B.** Dose-response curve for the inhibitory effect shown in part A. Inhibition was statistically significant at caffeine concentrations of 10^{-4} M and higher ($p < 0.01$). **C.** Inhibitory effect of caffeine at 1 and 10 mM in normal (empty columns) and Ca^{2+}-free solutions. 0% inhibition corresponds, in each CB, to the control value previous to the application of caffeine (2.13 ± 0. 32 % from total ^{3}H-CA content). There were no statistical differences between 1 and 10 mM effects in the presence or absence of Ca^{2+} or between the two groups.

A full dose-response curve for this effect in Ca^{2+}-containing solutions showed that the caffeine concentration threshold required to inhibit basal ^{3}H-CA release was 10^{-5} M. Overall, the curve fitted a sigmoid with an IC_{50} of 210 μM, and maximal inhibition reached 55.8% (Figure 28B).

Since a common target of caffeine in many cell types are ryanodine receptors located in the endoplasmic reticulum, and the action of caffeine on this target is Ca^{2+}-dependent, we also tested caffeine in Ca^{2+}-free solutions.

Figure 28C compares mean percentage inhibition of the basal release produced by 1 and 10 mM caffeine in normal and 0 Ca^{2+}-containing solutions obtained in a group of 4 CBs for each concentration. The absence of statistical differences would indicate a minimal or no contribution of intracellular Ca^{2+} deposits to the inhibitory effect of caffeine.

5.3.2. Effect of caffeine on the release of ^{3}H-CA induced by hypoxia and high external K^+

In next series of experiments we tested caffeine for its effect on the release of ^{3}H-CA elicited by natural hypoxic stimuli of mild and severe intensities (10 min incubation with solutions equilibrated with 10 and 5% O_2; $PO_2 \approx$ 66 and 33 mmHg, respectively), and by a depolarising stimulus, high extracellular K^+ (2 min incubation with 30 and 50 mM K^+).

In both cases caffeine (1mM) was present in the incubating solutions prior to and during the application of the stimuli. Figure 29 shows the data for the hypoxic stimuli. Figures 29A and B present mean time courses of the ^{3}H-CA release for both intensities of stimulation, and in both cases the inhibitory effect of caffeine on the basal release is evident. However, although caffeine was very effective in inhibiting the release evoked by the mild hypoxia, it did not statistically change the release response elicited by the stronger hypoxic stimulus. Figure 29C shows the mean evoked response (equivalent to the area under the curve in panel A): caffeine diminished the release of ^{3}H-CA evoked by mild hypoxia from 2.02 $\pm$ 0.20 (n = 9) to 0.79 $\pm$ 0.21 (n =7) percent of tissue content, equivalent to an inhibition of the release response of a 69% ($p < 0.01$), and did not alter the response elicited by the stronger hypoxic stimulus (7.80 $\pm$ 1.62 (n= 9) in control CBs and 9.91 $\pm$ 1,23 (n =8) in the presence of caffeine; $p > 0.05$).

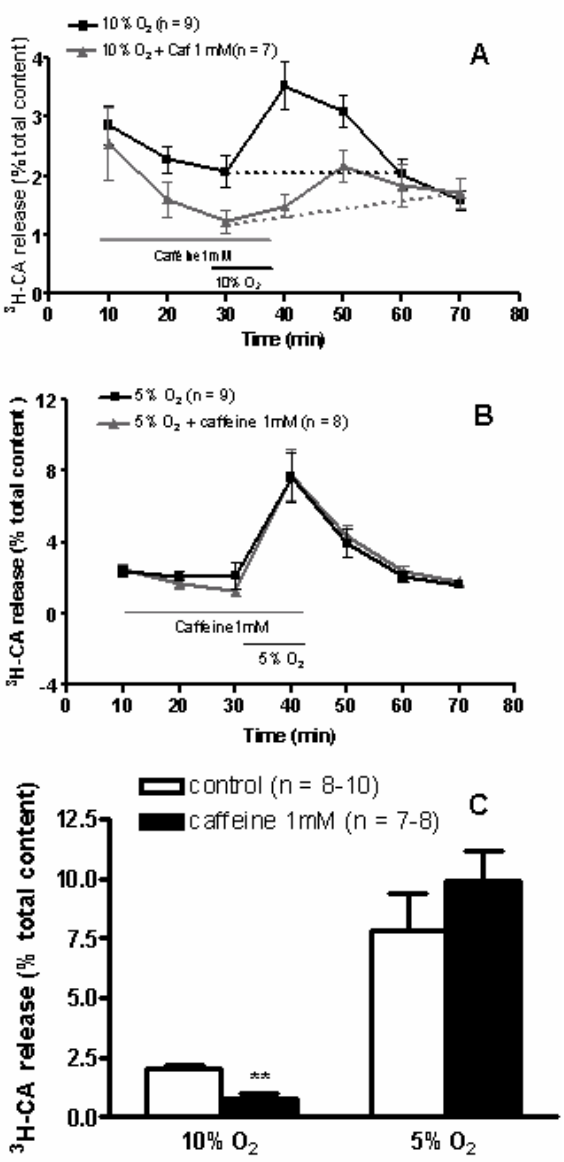


Figure 29 Effect of 1mM of caffeine on the release of CA from rat carotid body induced by hypoxia. **A** and **B** show the time courses for the release of ^{3}H-CA in response to a 10 min incubation with solutions equilibrated with 10 and 5% O_2, respectively. Caffeine was applied 20 min prior to as well as during the 10 min of the hypoxic stimulation. Note also the inhibitory effect of caffeine on the basal release of ^{3}H-CAs. In part A, the dotted lines going from the control fraction prior to the application of the hypoxic stimulus to the post-stimulus period when the release returned to basal, define the stimulus induced release of ^{3}H-CA. To calculate the release as percentage of the total tissue content, the dpm as ^{3}H-CA in each collected fraction (or the evoked release) were divided by the dpm of ^{3}H-CA present in the tissue at any given moment in the experiment (i.e. dpm present in the tissue at the end of the experiment plus dpm present in the fractions collected after the one under consideration). **C.** Mean (± SEM) evoked release by 10 and 5% O_2. **p<0.01; two-way ANOVA with Bonferroni multi-comparison test.

An equivalent group of experiments performed with high levels of external K^+ of moderate and strong intensities yielded comparable results (Figure 30) i.e. in the time course of the experiments with both concentrations of K^+ the inhibitory effect of caffeine on basal release is evident (Figure 30A and B). The inhibition of the release induced by 30 mM K^+ (Figure 30A) and the lack of statistically significant effect of caffeine on the response elicited by 50 mM K^+ (figure 30B B) is also evident. Mean inhibition produced by 1 mM of caffeine on the response elicited by 30 mM K^+ amounted to 64% (Figure 30C; $p < 0.01$). Increasing the concentration of caffeine to 10 mM did not augment the inhibition on the release elicited by hypoxic and high K^+ stimulus (not shown). These

findings indicate that the inhibitory effect of caffeine on the release of CA from CB depends on the intensity of the applied stimulus.

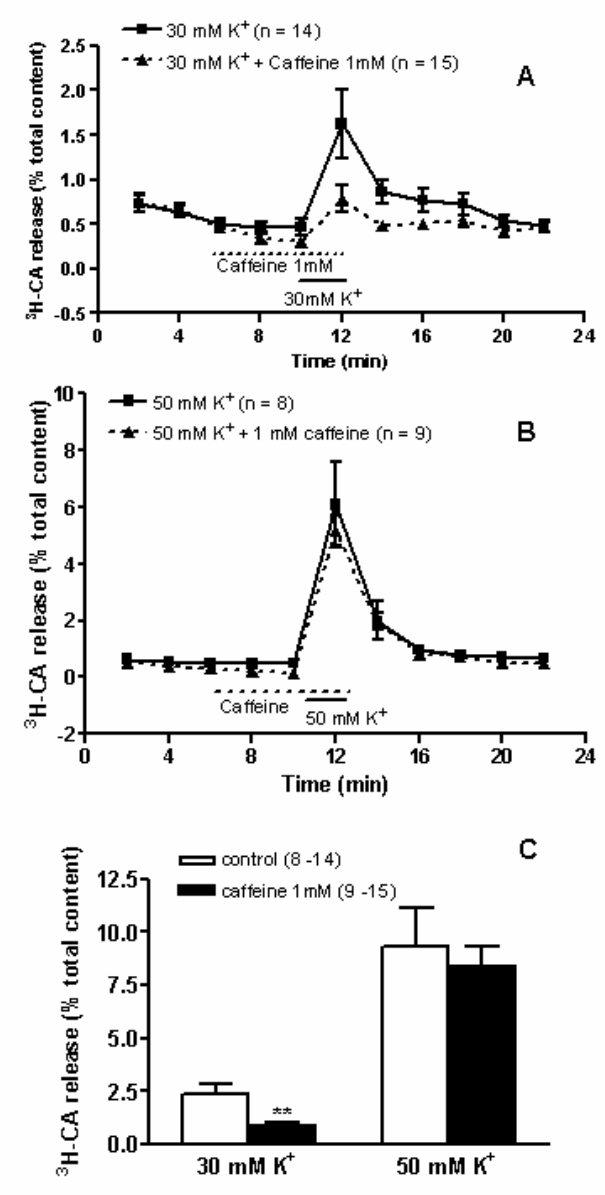


Figure 30 Effect of 1mM of caffeine on the release of CAs from rat carotid body induced by 30 and 50 mM of extracellular K^+. **A** and **B** show time courses of the release of ^{3}H-CA. Caffeine and high K^+ were present in the incubating solutions at the times marked in the drawing. Note that with high K^+ as stimulus the incubating solutions were collected for their analysis in ^{3}H-CA content every 2 min. Also note that in spite of the large ordinate scale in this figure, still is possible to observe the inhibitory effect of caffeine on the basal, pre-stimulus samples. **C**. Mean (± SEM) evoked by 30 and 50 mM extracellular K^+. **p<0.01; two-way ANOVA with Bonferroni multi-comparison test.

5.3.3. Pharmacological characterisation of adenosine receptors involved in the inhibitory effect of caffeine on the release of ^{3}H-CA from the carotid body

The Ca^{2+} independence of the effect of caffeine on the basal release and its range of effective concentrations suggested that caffeine effects were mediated via adenosine receptors (Fredholm et al., 1999). Therefore we

investigated the type(s) of adenosine receptors involved in the inhibitory action caffeine.

First we tested NECA (1 μM), an A_2 agonist, on basal and low PO_2-induced release of ^{3}H-CA. Figure 31 shows the results. Figure 31A shows the time course of the release in preparations continuously incubated with 20% O_2 or with 10% O_2. First, notice that the time course is similar in both incubating conditions, except for the higher rate of release with the lower PO_2, and second, notice that in both cases NECA produced an increase in the release of ^{3}H-CA that returned to prior time course after removal of the drug. Figure 31B shows mean effects obtained in groups of 3-6 CB. At both PO_2, NECA increased the ongoing release of ^{3}H-CA, the increase being higher in normoxia but not significantly different (P=0.13) than in moderate hypoxia amounting to a 54% and a 25% increase over the correspondent control, respectively.

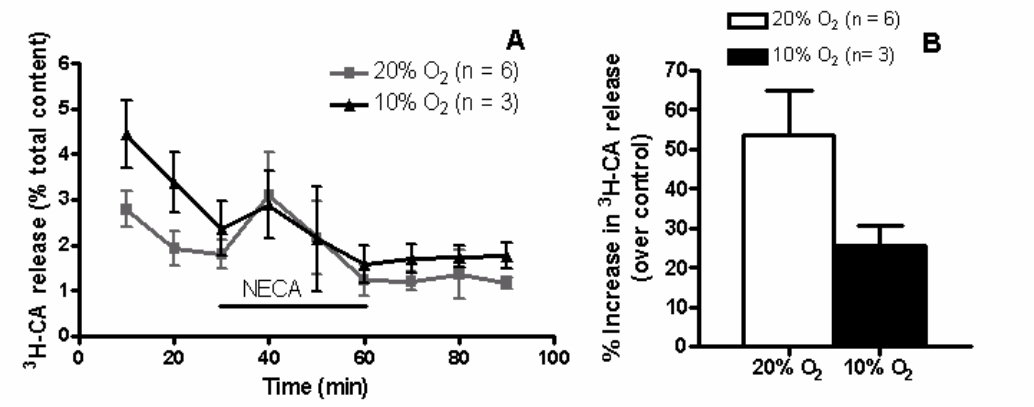


Figure 31 Effects of NECA, an A_2 adenosine receptor agonist, on the release of ^{3}H-CA from rat carotid body. **A.** Time courses of the release of ^{3}H-CA from CBs incubated with 20% (normoxic) and 10 % O_2 (hypoxic) solutions. NECA (1 μM) was applied as shown in the drawing. **B.** Percentage increase in the release of ^{3}H-CA elicited by NECA. Data represent means ± SEM.

In a second group of experiments presented in Figure 32A (and 32B) we intended to reverse the effects of caffeine on basal release of ^{3}H-CA with two A_{2A} agonists, CGS 21680 and HE-NECA. From both figures it is evident that neither of the agonists altered the ongoing time course of the release, and also, that neither of them prevented the inhibition of the release produced by caffeine. Complementary experiments on the possible involvement of A_{2A} receptors are shown in Figures 32C and 32D. When SCH 58621 (20 and 200 nM), a specific

antagonist of A_{2A} adenosine receptors, was tested on the release of ^{3}H-CA elicited by 30 mM K^+ it was evident that it altered neither the time course (Figure 32C) nor the magnitude (Figure 32D) of the release response.

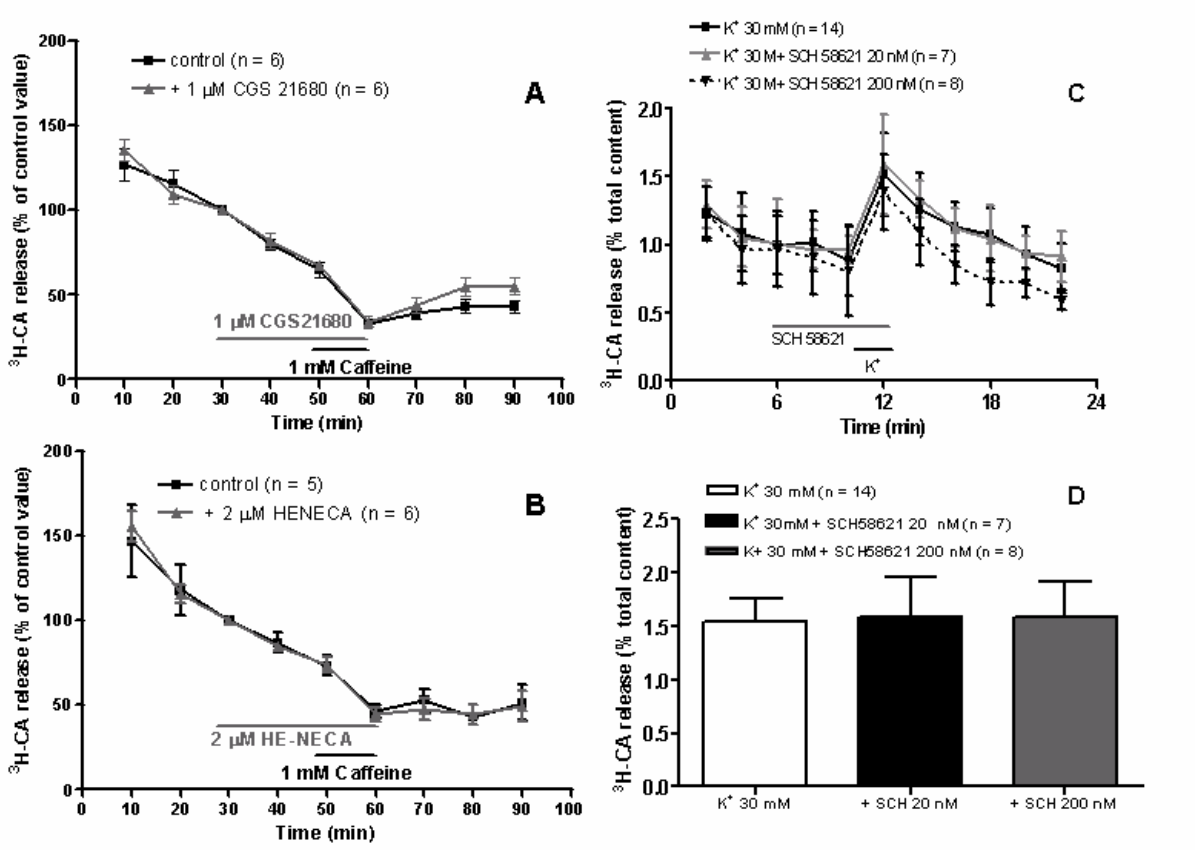


Figure 32 Effect of A_{2A} adenosine agonists and antagonists on the release of ^{3}H-CA and on the inhibition of the release produced by caffeine in the rat CB. **A** and **B**, CGS 21680 (1 μM) and HE-NECA (2 μM), two A_{2A} adenosine agonists, did not alter basal release nor modify the inhibition of the release produced by caffeine (1 mM). Data are normalised to the release of ^{3}H-CA present in the samples immediately prior to the introduction of any drug to emphasise the parallelism between control (drug free) and experimental data. 100% was in all cases the release obtained immediately prior to the application of the drugs **C.** Effect of SCH58621, an A_{2A} adenosine receptor antagonist, on the time course of the release of ^{3}H-CA evoked by 30 mM K^+. High K^+ and SCH 58621 were present in the incubating solutions at the concentrations and times marked in the drawing. **D.** Mean evoked release elicited by 30 mM in control conditions and in the presence of 20 and 200 nM SCH 58621. Data represent means ± SEM.

Following identical protocols we studied the effects of two other adenosine receptor antagonists, DPCPX and MRS 1754. DPCPX at low concentrations (low nM) is an antagonist of A_1 adenosine receptors, but at higher concentrations it also interacts with A_{2B} adenosine receptors (Fredholm

et al., 2001). Consistent with this profile of affinities, when DPCPX was applied at a 150 nM concentration (thereby preferentially inhibiting A_1 adenosine receptors), it produced a non-significant tendency to inhibit the release of ^{3}H-CA evoked by 30 mM of extracellular K^+ in the CB. However, at a concentration 10 times higher (1.5 μM) it inhibited the release of ^{3}H-CA elicited by the same stimulus by 58.96% (Figure 33A). MRS 1754, a specific A_{2B} adenosine receptor antagonist, inhibited the release of ^{3}H-CA elicited by 30 mM in a dose dependent fashion, reaching the maximal inhibition of 51.48 and 52.44% at concentrations of 20 and 200 nM, respectively (Figure 33B).

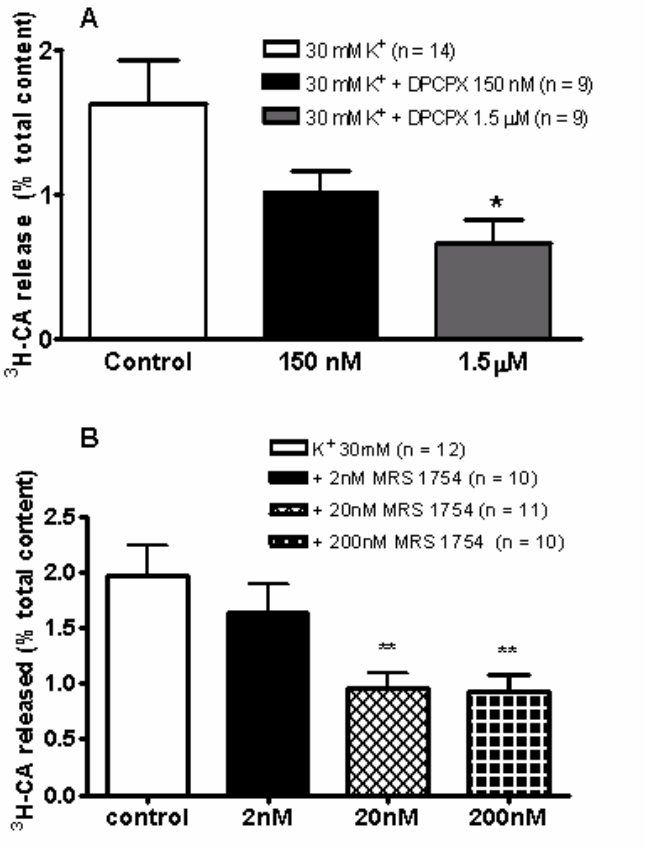


Figure 33 Effect of DPCPX (an A_1 and A_{2B} antagonist, **A**) and MRS1754 (an A_{2B} antagonist, **B**) on the release of ^{3}H-CA evoked by 30 mM extracellular K^+ in the rat carotid body. Experimental protocols as in previous figure. DPCPX was used at concentrations of 0.15 and 1.5 μM; only the higher concentration produced a significant inhibition of the high K^+ evoked release of ^{3}H-CA. MRS1754 was used at concentrations of 2, 20 and 200 nM; the highest concentrations produced a nearly identical inhibition of the high K^+ evoked release of ^{3}H-CA. Data represent means ± SEM. * p< 0.05, **p< 0.01; one-way ANOVA with Dunnett's multi-comparison test.

The findings with CGS 21680, HE-NECA and SCH 58621 indicate that A_{2A} receptors do not mediate the inhibition of the release of ^{3}H-CA produced by caffeine. On the other hand, the effect of NECA and the fact that the percentage of inhibition attained with caffeine, DPCPX at high concentration and MRS 1754 are comparable would indicate that the inhibitory action of caffeine is mediated by A_{2B} receptors located in chemoreceptor cells.

5.3.4. Immunocytochemical demonstration of A_{2B} adenosine receptors in chemoreceptor cells

Previous studies from Kobayashi et al. (2000a) have shown the expression of the gene for A_{2B} adenosine receptors in homogenates of rat CB, but their location at the cellular level was not established. As our results strongly suggest that the location of this subtype of receptors is in chemoreceptor cells, we performed immunocytochemical studies aimed to confirm this suggestion. Figure 34 shows that indeed A_{2B} receptors co-localize in fresh cultures of dissociated CB with TH, a specific marker for chemoreceptor cells. In Figure 34A there is a bright field image of a 20 h old culture; Figures 34B and C show, respectively, TH^+ chemoreceptor cells and A_{2B} receptor positive cells in the same microscopic field; Figure 35D shows the image resulting from superimposition of images in panels B and C.

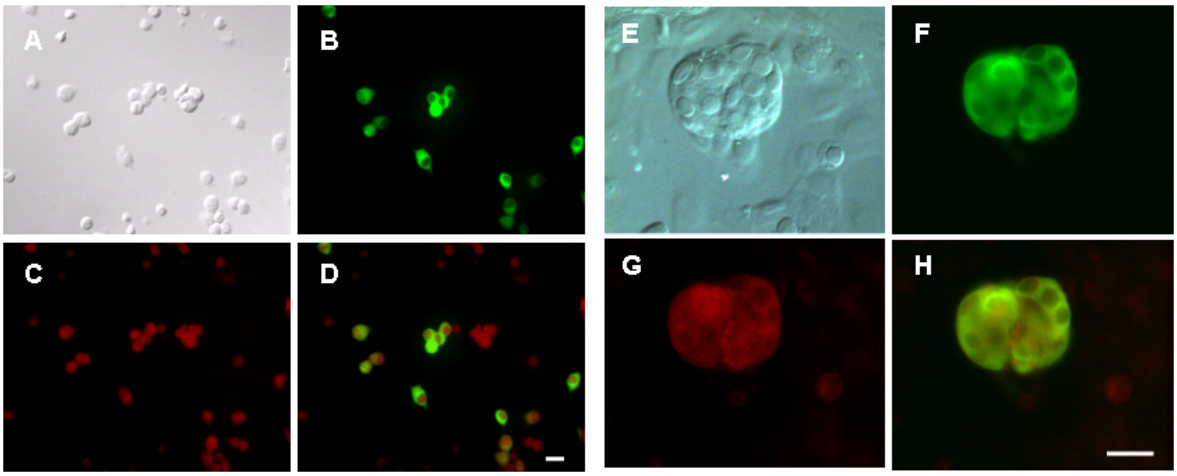


Figure 34 Immunocytochemical demonstration of A_{2B} adenosine receptors in dissociated CB cells in culture. **A-D** correspond to a microscopic field of a culture with fully dispersed cells. A shows the bright field image, B shows the cells that are immunopositive for tyrosine hydroxylase representing chemoreceptor cells, C shows the cells that are immunopositive for A_{2B} receptors and D shows the image of B and C merged. **E-H** correspond to a microscopic field containing a glomoid from a partially dispersed CB in culture. Images show an identical distribution to that in the prior series. Calibration bars, 20 μm.

Overall it is evident that all TH^+ chemoreceptor cells are also positive to A_{2B} receptors. In addition, many TH^- cells that are immunoreactive to A_{2B} receptors are present, as well as some cells that are unreactive to either

antibody. Panels E to H of Figure 34 show images obtained from a culture in which the mechanical dispersion during the dissociation of the CB was minimised to obtain complete clusters of chemoreceptor cells. Once again it is evident that the entire glomoid is positive to TH and to A_{2B} receptors, with some dispersed cells also present, that being negative to TH, are positive or negative to A_{2B} receptors.

5.3.5. Actions of caffeine on the carotid sinus nerve activity: a mixed A_{2A} and A_{2B} receptor mediated effect

The next group of experiments was designed to assess the effect of caffeine on the overall output of the CB measured as chemosensory activity (action potential frequency) in the CSN. Figure 35 shows the results obtained when caffeine was used at a concentration of 10 mM. In Figure 35A it is evident that caffeine markedly inhibited the hypoxic (perfusion with a 5% O_2-equilibrated solution) augmentation of the CSN action potential frequency, without apparently modifying the basal normoxic activity of the CSN. Mean inhibition of the peak hypoxic response was 50.40% as the ΔCSN frequency reached 82.91 $\pm$ 13.53 (n=9) impulses/sec in the control preparations and only 41.78 $\pm$ 9.32 (n=8) in the preparations superfused with the same hypoxic solution containing 10 mM caffeine (Figure 35B); caffeine did not modify basal activity. Caffeine did not delay the onset of the response (latency of the response) but significantly augmented the time to reach maximal activity (time to peak; Figure 35C). Sample neurograms obtained at different moments of the experiment marked in Figure 35 are shown at the top of that Figure.

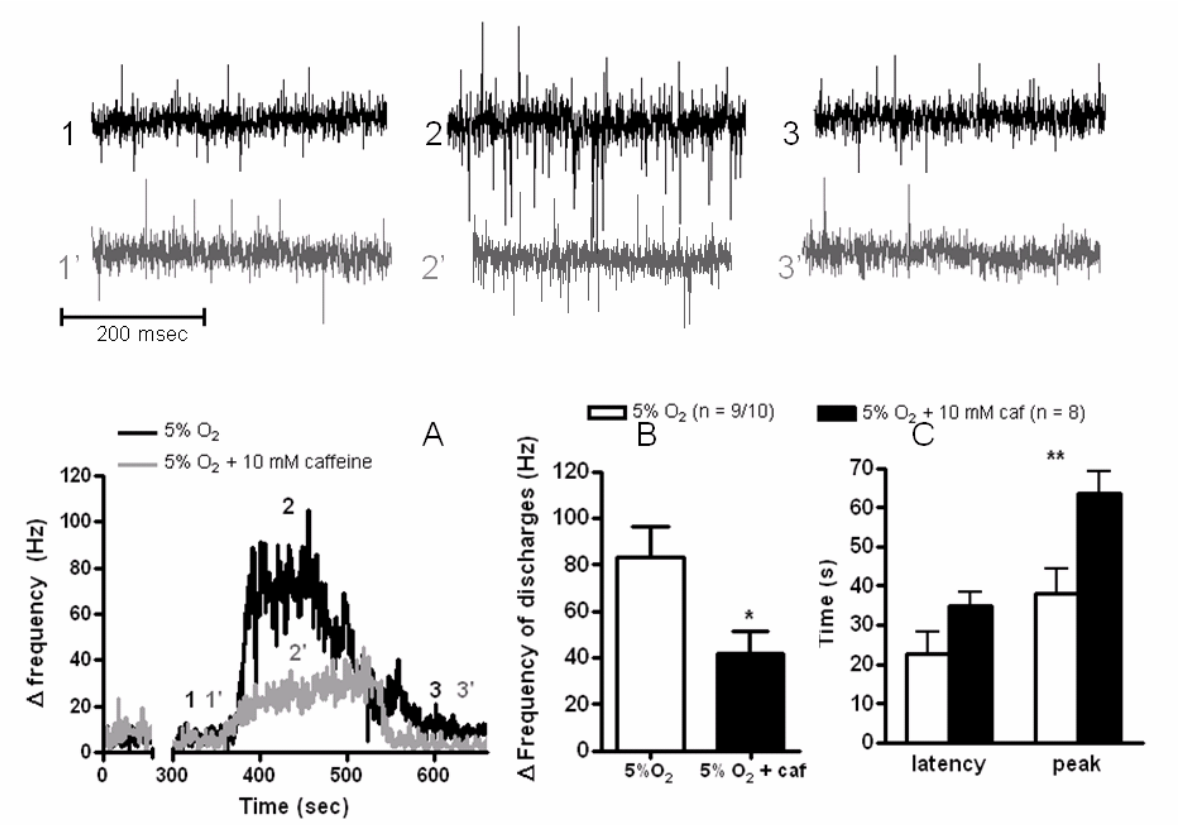


Figure 35 Effect of caffeine on the carotid sinus nerve activity (CSN) elicited by hypoxia (5% O_2). **A**. Typical recording of the effect of caffeine (10 mM) on the frequency of CSN action potentials during superfusion with a solution equilibrated in response to 5% O_2. The control response to hypoxia is shown in black and the effect of caffeine on the hypoxic response in grey; the responses were obtained from the same CB and caffeine was present in the superfusing solution for the 5 min prior to and the 3 min of hypoxic stimulation. Sample neurograms obtained at the times indicated in A are shown at the Figure. **B** and **C** show, respectively, mean peak frequencies and latencies and times to peak, in control (empty bars) and in caffeine-treated preparations (black bars). Data represent means ± SEM. *p< 0.05, Student's *t* test; **p < 0.01; two-way ANOVA with Bonferroni multi-comparison test.

As maximal inhibition of the CSN activity produced by 10 mM caffeine was comparable to the inhibition of the basal as well as mild hypoxic stimulus and 30 mM K^+ induced release of ^{3}H-CA, we tested whether the full dose response curve was also comparable i.e. we tested if the entire inhibitory action of caffeine on CSN activity could be attributed to its presynaptic effect via A_{2B} receptors. We found (Figure 36A) that the action of caffeine on the CSN elicited by hypoxia has two clear components, one of low affinity for caffeine produced at the same range of concentrations affecting the release of ^{3}H-CA, and the other of high affinity for caffeine, thereby produced by much lower

concentrations of the drug. In fact, when the full dose-response curve was analysed it was found that it could be defined by the sum of two Michaelis-Menten equations:

$$\%Inhibition = \{(I_{max1} \bullet [Caff])/(K_{m1} + [Caff])\} + \{(I_{max2} \bullet [Caff])/(K_{m2} + [Caff])\}$$

The high affinity component had a K_m (K_{m1}) 17.90 x 10^{-9} M and produced a maximal inhibition (I_{max1}) of 29.81%. The low affinity component had Km and Imax of 160 x 10^{-6} M and 21.35%, respectively. The resulting maximal effect of caffeine on the CSN activity elicited by hypoxia was 51.16%. Interestingly enough the K_m of the low affinity component is quite similar to that found for caffeine at the presynaptic level (210 x 10^{-6} M; Fig. 28B). Comparable IC_{50} (11.78 nM and 171.0 μM; r = 0.999) were obtained if the data were fitted to a two-site competition curve using the Graph Pad Prism Software.

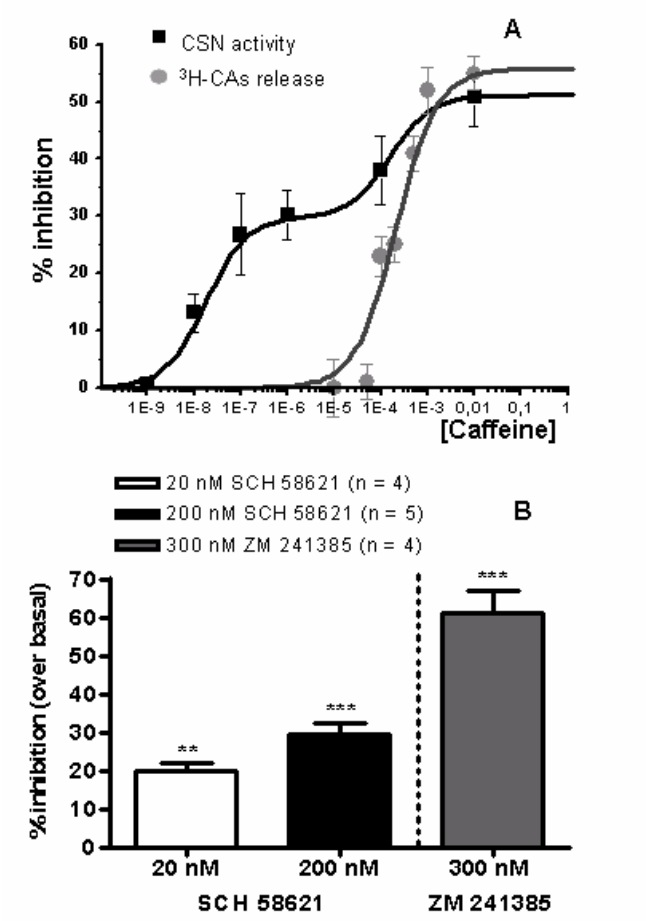


Figure 36 Kinetic and pharmacological analysis of the effects of caffeine on the carotid sinus nerve response to hypoxia (5% O_2). **A** shows, in black, the dose-response curve for caffeine inhibition of the chemosensory activity elicited by hypoxia in the carotid sinus nerve, and, superimposed in grey, the effects of caffeine on the release of ^{3}H-CA. **B.** Percent inhibition of the response elicited by hypoxia in the carotid sinus nerve produced by an A_{2A} antagonist (SCH 58621) and a mixed A_{2A} and A_{2B} antagonist (ZM 241385). Data represent means ± SEM. **p< 0.01; ***p < 0.001; one-way ANOVA with Dunnett's multi-comparison test.

A pharmacological analysis of the effect of caffeine on the activity of the CSN (Figure 36B) revealed that the high affinity component of the caffeine effect was mediated by A_{2A} adenosine receptors, because SCH 58621 at concentrations of 200 nM (over 10 times higher than its Kd for A_{2A} receptors) inhibited hypoxic CSN activity by 29.53 ± 2.73% (n= 5). The mixed A_{2A} + A_{2B} antagonist, ZM 241385, at concentrations of 300 nM (nearly 75 times higher than the Kd for A_{2A} and 10 times the Kd for A_{2B}) produced an inhibition of the CSN hypoxic activity of 61.22 ± 5.83%, which is comparable to the maximal inhibition produced by caffeine (n=4). This last result suggests that the low affinity component of caffeine action on CSN activity is mediated by A_{2B} receptors.

5.4. Discussion

Using a combination of neurochemical, pharmacological and immunocytochemical techniques we have demonstrated the presence of A_{2B} receptors in chemoreceptor cells, demonstrating that they control the capacity of the cells to release neurotransmitters, assessed as the release of CA, in conditions of normoxia and low intensity of stimulation. We have also shown that the chemosensory activity elicited by hypoxia in the CSN is controlled by adenosine, with this last effect mediated by a cooperative action between A_{2A} and A_{2B} receptors.

At the outset of the discussion we would like to point out that the release of CA has been used exclusively as an index of the chemoreceptor cell activity (Fidone et al., 1982; Gonzalez et al., 1992; Montoro et al., 1996) without any implication on its significance regarding the genesis of the activity in the CSN. In fact, there are now two laboratories that have shown a lack of parallelism between CA release and the CSN chemosensory responses (Donnelly, 1996; Iturriaga et al., 1996; 2000). Furthermore, since we have been measuring the actions of adenosine receptor antagonists throughout our study, our findings suggest that adenosine is being endogenously released and therefore our observations result from the inhibition of its physiological action.

In accordance with these statements, our observation that caffeine, a non-specific adenosine receptor antagonist, inhibits normoxic release of CA as

well as the release elicited by stimuli of low intensity, would imply that in these conditions we are preventing the actions of adenosine that is concomitantly released, and therefore, that the physiological effect of adenosine is an activation of the release of CA acting on A_{2B} receptors (see below). Therefore, in the rest of the discussion we will refer in an undifferentiated fashion to the inhibitory effect of caffeine or the excitatory effect of adenosine. It is worth noting that there is a significant normoxic release of endogenous adenosine and that the peak release is attained at low levels of hypoxic stimulation (superfusion with 10% O_2) decreasing with higher intensities of hypoxic stimulation (Chapter 1 of this work; Conde and Monteiro, 2006). This pattern of adenosine release would explain our observation that the A_2 agonist, NECA, seems to be more effective in activating the release of ^{3}H-CA in normoxia than in moderate hypoxia, when adenosine receptors would be more occupied by endogenous adenosine.

In spite of the fact that the pharmacological analysis we have performed substantiates that caffeine effects are mediated via adenosine receptors, we would like to point out that literature and our previous observations also support this contention. Thus, the concentrations at which caffeine produces its effects on the release of CA in the CB are below those required to activate ryanodine receptors (Xu et al., 1998), and, in addition, the usual effect of caffeine acting via ryanodine receptors is an activation of the Ca^{2+}-dependent responses, including the release of neurotransmitters and hormones (Johnson et al., 2000). In this context it should be noted that neither thapsigargin nor ryanodine, which also act on intracellular Ca^{2+} deposits, were capable of modifying the release of CA in rat chemoreceptor cells or the release of CA and intracellular free Ca^{2+} in rabbit chemoreceptor cells (Vicario et al., 2000b; Conde et al., 2006a). At the concentrations used, caffeine might inhibit phosphodiesterases and thereby promote an increase in cAMP levels (Fredholm et al., 1999), however the expected effect of caffeine acting at this target would be an increase in the release, particularly during hypoxic stimulations of any intensity (Perez-Garcia et al., 1991; Chen et al., 2000). Our pharmacological analysis indicates that caffeine inhibition of the release of CA is mediated by A_{2B} receptors. In our analysis we have selected concentrations of all agents in such a way that at least one of the concentrations tested is $\geq$10 times higher than the described Kd

for the agents (www.iuphar.org), and therefore we are confident that both the inhibition or stimulation of each receptor subtype was maximal. Consistent with the pharmacological data, our immunocytochemical studies demonstrate, for the first time, that chemoreceptor cells express A_{2B} receptors. It should be noted, however, that for every cell identified as a chemoreceptor by their being positive to tyrosine hydroxylase, there are many other cells in the primary cultures of the CB that also express A_{2B} receptors, confirming the PCR studies of Kobayashi et al. (2000) that show a high level of receptor expression in whole CB homogenates. It should mentioned, however, that this inhibitory effect of caffeine on rat CB chemoreceptor cells seems to be species specific since in previous studies performed in rabbit, cat and kitten CBs, this xanthine altered neither the basal release of CA from CB (Vicario et al., 2000b) nor the CSN neural discharge (Bairam et al., 1997).

The fact that caffeine (and the other agents tested) produces identical effects in the response elicited by high K^+ (a non-specific depolarising stimulus) and hypoxia would suggest that A_{2B} receptors are coupled towards the last steps in the stimulus-secretion coupling. Thus, it is generally accepted that in chemoreceptor cells the stimulus-secretion coupling in response to hypoxic stimulus occurs according to following steps (Gonzalez et al. 1992; see also Lahiri et al., 2006): (1) O_2-sensing at an O_2-sensor (not fully identified) → (2) activation of coupling mechanisms with K^+ channels → (3) change in kinetics of these K^+ channels resulting in a decrease in their opening probability → (4) cell depolarisation → (5) activation of voltage operated channels → (6) Ca^{2+} entry and increase in intracellular free Ca^{2+} → (7) activation of exocytosis and neurotransmitter release. As the transduction of high K^+ into the exocytosis of neurotransmitters follows the same path starting at step (4), the modulatory action of adenosine should occur at between steps 4 and 7. However, clarification of the intimate mechanisms of adenosine (caffeine) actions on the exocytotic process would require additional experiments.

The last group of our findings relates to the effects of caffeine on the chemosensory activity in the CSN. The pharmacological profile of caffeine effects on the chemosensory activity in the CSN is completely different to that observed upon the release of CA. The most important corollary of our observation that caffeine can inhibit the activity in the CSN is that endogenously

released adenosine is cooperating positively in the genesis of the overall output of the CB in situations of hypoxia. An intriguing observation is that caffeine, at any of the concentrations tested from 1x 10^{-9} to 10^{-2} M, did not affect normoxic ongoing activity in the CSN; the puzzle arises when this observation is compared with those made upon the release of CA. Another clear difference in the effects of caffeine at the presynaptic (release of CA) and postsynaptic level is the dose-response curve: 10000 times lower caffeine concentrations than those required to produce an effect at the presynaptic level were able to significantly reduce the activity in the CSN elicited by hypoxia. In addition, there were two clear components in the inhibitory action of caffeine on the CSN activity, one with a very high affinity (IC_{50} 17.9 nM) and another with low affinity (160 μM), the high-affinity component being mediated by A_{2A} receptors and being responsible for ≈60% of the total inhibition, and the low-affinity component being mediated by A_{2B} receptors and being responsible for the remaining ≈40%. As noted above in the description of our findings, the IC_{50} of this lower component is nearly identical to the IC_{50} of caffeine on the release of CA. Based on these observations we would suggest that the high-affinity component of the CSN inhibition might be produced by direct action of caffeine on the sensory nerve endings themselves, with the low-affinity component being the result of the presynaptic action of caffeine. However, we are not implying that high concentrations of caffeine are inhibiting the activity in the CSN because they inhibit the release of CA; it is well known that many other neuroregulators (e.g. ATP) are co-released with CA (Kupfermann, 1991). Nor are we implying that the only mechanism for the high-affinity component of the inhibitory action of caffeine is a direct action on A_{2A} receptors located in the sensory nerve endings: the possibility exists that adenosine via A_{2A} receptors inhibits the high conductance anion channel which appears to represent one of the main pathways for ATP release (Sabirov and Okada, 2004) that is known to be expressed in chemoreceptor cells (Stea and Nurse, 1989). However, all these suggestions await future experimental support. A final consideration in this discussion on the identity of the receptors involved in the effects of adenosine relates to the evident species differences. In a prior study by Prof. C. Gonzalez's laboratory (Rocher et al., 1999) it was shown that adenosine acting

via A_1 receptors inhibits Ca^{2+} currents and the release of catecholamines in rabbit chemoreceptor cells. We also want to point out that there are two recent studies in rat chemoreceptor cells with opposing views on the action of adenosine on intracellular Ca^{2+} levels: Kobayashi et al. (2000a) demonstrated that adenosine did not alter $[Ca^{2+}]_i$ in cells exposed to normoxia, while Xu et al., (2006) showed that adenosine via A_{2A} receptors triggers a small increase in intracellular Ca^{2+} levels. However, the increase in the intracellular Ca^{2+} observed by Xu et al. (2006) seems to be insufficient to reach the threshold to evoke a release of neurotransmitters (Vicario et al., 2000b).

Together our results indicate that caffeine has an inhibitory effect on peripheral control of breathing. However, in pre-term infants caffeine has an overall stimulating effect on ventilation (Comer et al., 2001). Because it is known that caffeine acting at the brain stem respiratory neurons stimulates ventilation (Herlenius and Lagercrantz, 1999; Wilson et al., 2004) the inhibitory effects on peripheral control of ventilation described herein would indicate that the central effects are dominant in infants. The dominance of the central effects in newborns could result from the immaturity of the CB at these early stages of life (for a review see Gauda et al., 2004b). In adults, where the function of the CB is fully expressed, the peripheral inhibitory effect of caffeine would be dominant, and inhibition of ventilation would result (Howell and Landrum, 1995). This would be particularly noticeable when the ventilation drive depends mostly on CB chemoreceptors, as happens in hypoxia.

In conclusion, chemoreceptor cells express A_{2B} receptors, which physiologically contribute towards fixing the level of neurotransmitter release in normoxic and low intensity stimulation conditions. Acting at this presynaptic site adenosine significantly ($\approx$ 25%) contributes to the CSN activity observed during hypoxic stimulation. The CB also expresses A_{2A} receptors, presumably expressed in the sensory nerve endings of the CSN, which appear to be responsible for nearly 30% of the activity elicited by hypoxia. Overall our findings provide an explanation for the observed inhibitory action of caffeine on hyperventilation produced by hypoxia.

6. CHAPTER 4 – ADENOSINE MODULATES THE RELEASE OF CATECHOLAMINES FROM RAT CAROTID BODY CHEMORECEPTORS THROUGH AN INTERACTION BETWEEN D_2 DOPAMINE RECEPTORS AND A_{2B} ADENOSINE RECEPTORS

6.1. Introduction and aim

In Chapter 3 (Section 5) we demonstrated that adenosine modulates the release of CA from rat CB in basal conditions and in response to low intensity stimulus via an action on A_{2B} adenosine receptors. Some previous data on CSN activity and ventilation *in vivo* have also suggested that DA and adenosine interact in the CB (Monteiro and Ribeiro, 2000). We postulated that this modulatory effect could occur due to an interaction between adenosine A_{2B} and dopamine D2 receptors present in glomus cells or intracellularly at the second messengers' level. Interactions between A_{2A}-D2 receptors are well described in the central nervous system (see General Introduction (Section 1.5.2.3.)) and these findings prompted us to investigate if interactions between A_{2B} and D2 receptors could be involved in the modulation by adenosine of CA release in the CB using a pharmacological and functional approach.

6.2. Materials and methods

6.2.1. Animals and surgical procedures

Experiments were performed in adult Wistar rats of both sexes (250-350 g) obtained from the vivarium of the Faculty of Medicine of the University of Valladolid. The Institutional Committee of the University of Valladolid for Animal Care and Use approved the protocols. All the surgical procedures were performed as previously described in Section 5.2.1.

For the release of CA experiments (5-12 CBs/experiment) the CBs were cleaned free of CSN and nearby connective tissue following procedures previously described (Vicario et al., 2000a).

6.2.2. Labelling of catecholamine stores: release of ^{3}H-CA

To monitor the release of CA a radioisotopic method was used, as described by Fidone et al. (1982) and Vicario et al. (2000a), and detailed in Section 5.2.2.. Specific protocols for stimulus and drug applications are provided in the Results. Stimulus included hypoxia with 10% O_2-equilibrated solutions and high external K^+ (30 mM) The agonists and antagonists used were: caffeine (a non-selective adenosine antagonist, 1mM), domperidone (a D_2 dopamine antagonist, 0.5 nM - 5 μM), haloperidol (a D_2 dopamine antagonist, 0.01-10 μM), 5'-(N-ethylcarboxamido)adenosine (NECA, an adenosine A_2 agonist; 0.1-100 μM), propylnorapomorphine (a D_2 dopamine agonist, 0.02-200 nM) and sulpiride (a D_2 dopamine antagonist,1 μM). After incubation, the incubated solutions and the CBs themselves were analysed, as described in 5.2.2.

6.2.3. Drugs and chemicals

6-methyl-tetrahydropterine, ascorbic acid, caffeine, domperidone, haloperidol, NECA, propylnorapomorphine and sulpiride were all obtained from Sigma (Sigma-Aldrich, Madrid, Spain). ^{3}H-tyrosine was obtained from Amersham (Madrid). Domperidone, haloperidol, NECA and sulpiride were prepared as 5 mM stock solutions in DMSO; the final concentration of DMSO was always below 1/500 which by itself lacks effects on our preparations. Propylnorapomorphine was prepared in a 5 mM stock solution in methanol.

6.2.4. Data analysis

Data were evaluated using Graph Pad Prism Software, version 4 and were presented as means ± SEM. Dose-response curves were fitted to Sigmoidal dose-response curves and the IC_{50}, EC_{50} and the Emax values were obtained directly from the Sigmoidal dose-response equation. The significance of the differences between the means was calculated using unpaired Student's *t* test and by One and Two-Way Analysis of Variance (ANOVA) with Dunnett's

and Bonferroni multiple comparison tests, respectively. *P* values of 0.05 or less were considered to represent significant differences.

6.3. Results

6.3.1. Effect of NECA, an A_2 agonist on the release of ^{3}H-CA from rat carotid body

Caffeine at low concentrations is a non-selective antagonist of adenosine receptors (see Introduction, Section 1.7) that inhibits the basal and low intensity stimuli evoked release of ^{3}H-CA from CB, but not the release evoked by stimuli of higher intensity (Chapter 3, Section 5). Along the same experimental line, we have tested NECA, which is an agonist of A_2 adenosine receptors, on the basal, low intensity (10% O_2) (Figure 31, Section 5.3.3.) and high intensity hypoxia (2% O_2) evoked release of ^{3}H-CA from CB. In the previous experiments on the basal and low intensity stimulus evoked 3H-CA released, NECA was tested only in one concentration (1uM, see Fig 31) and the pharmacological characterisation of the A_{2B} subtype of receptors that modulate the release of ^{3}H-CA on chemoreceptor cells was performed using more selective agonists and antagonists (Chapter 5, Section 5.3.3.). Therefore, according to our statements that the receptor subtype that modulates the release of ^{3}H-CA on chemoreceptor cells is A_{2B} (Chapter 5, Section 5.3.3.) this would imply that the observed effects of NECA on the release of ^{3}H-CA are due to an action on A_{2B} adenosine receptors.

Figure 37 shows that the effect of NECA during normoxic conditions was concentration-dependent and that the increase in the basal release of ^{3}H-CA from rat CB had a maximal effect (E_{max}) of 105.3% and an EC_{50} of 1.22 μM (Figure 37A).

Previously, we have shown (Figure 31, Section 5.3.3.) that NECA also stimulates the release of ^{3}H-CA evoked by moderate hypoxia. Now we show that NECA, as was the case with caffeine, was not able to modify the release of ^{3}H-CA evoked by a higher intensity hypoxia, such as 2% O_2 (Figure 37B).

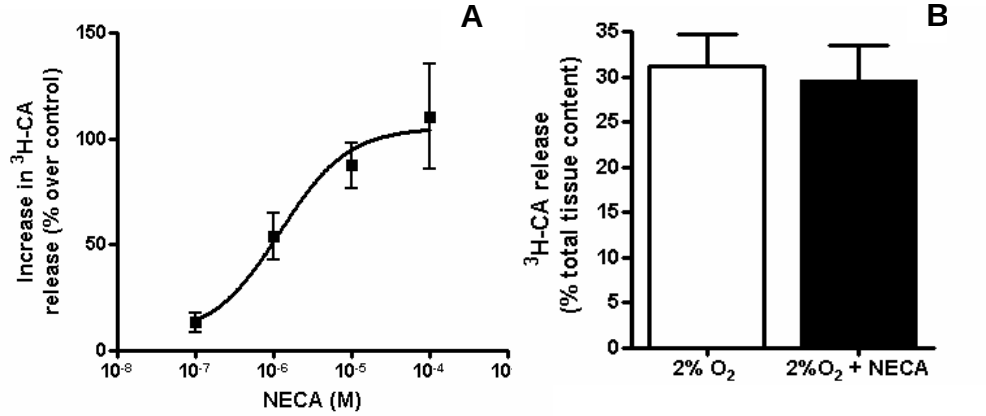


Figure 37 Effect of NECA, an adenosine A_2 agonist, on the basal release of ^{3}H-CA and on the release evoked by 2% O_2, from rat carotid body. A) Dose-response curve for NECA (0.1-100 μM) on the basal (normoxic, 20% O_2) release of ^{3}H-CA. 0% effect corresponds to 1.062 ± 0.06% of the total ^{3}H-CA content. B) Effect of 10 μM of NECA on the release of ^{3}H-CA evoked by 2% O_2. 0% corresponds to 1.669 ± 0.44 % of total ^{3}H-CA content. NECA was applied for 30 min between min 30 and 60 of the protocol in the experiments in basal conditions. In the experiments for the effect of NECA in hypoxia, the drug was applied in periods prior, during and after the hypoxic stimulation. Hypoxic stimulus was applied for 10 min. Data represent means ± SEM (n= 5-6).

6.3.2. Reversion of the inhibitory effect of caffeine on the ^{3}H-CA by a D_2 antagonist, sulpiride

Sulpiride (0.1-10 μM), which is a D_2 dopamine receptor antagonist, did not modify the basal release of ^{3}H-CA from rat CB (data not shown), but applied in a concentration of 1 μM, it produces an increase of 106.1 and 163.5% in the release of ^{3}H-CA evoked by 30 mM K^+ and 10% O_2, respectively (Figure 39A). The fact that sulpiride induced an increase in the release of ^{3}H-CA evoked by moderate hypoxia and 30 mM K^+ confirms the existence of D_2 dopamine autoreceptors in CB chemoreceptor cells that control the release of CA by a negative feedback mechanism. Despite the absence of effect of sulpiride on the basal release of ^{3}H-CA from CB, its effect on the evoked release of ^{3}H-CA by low intensity stimulus prompted us to investigate if this antagonist could abolish the inhibitory effect of caffeine (1 mM) on the evoked release of ^{3}H-CA from rat CB. It was observed that 1 μM of sulpiride almost completely abolished the inhibitory effect of caffeine on the basal response (data not shown) and on the release of ^{3}H-CA evoked by 30 mM K^+ (Figures 38B and 38C), suggesting that adenosine receptors interact with D_2 dopamine autoreceptors to control the release of CA from rat CB.

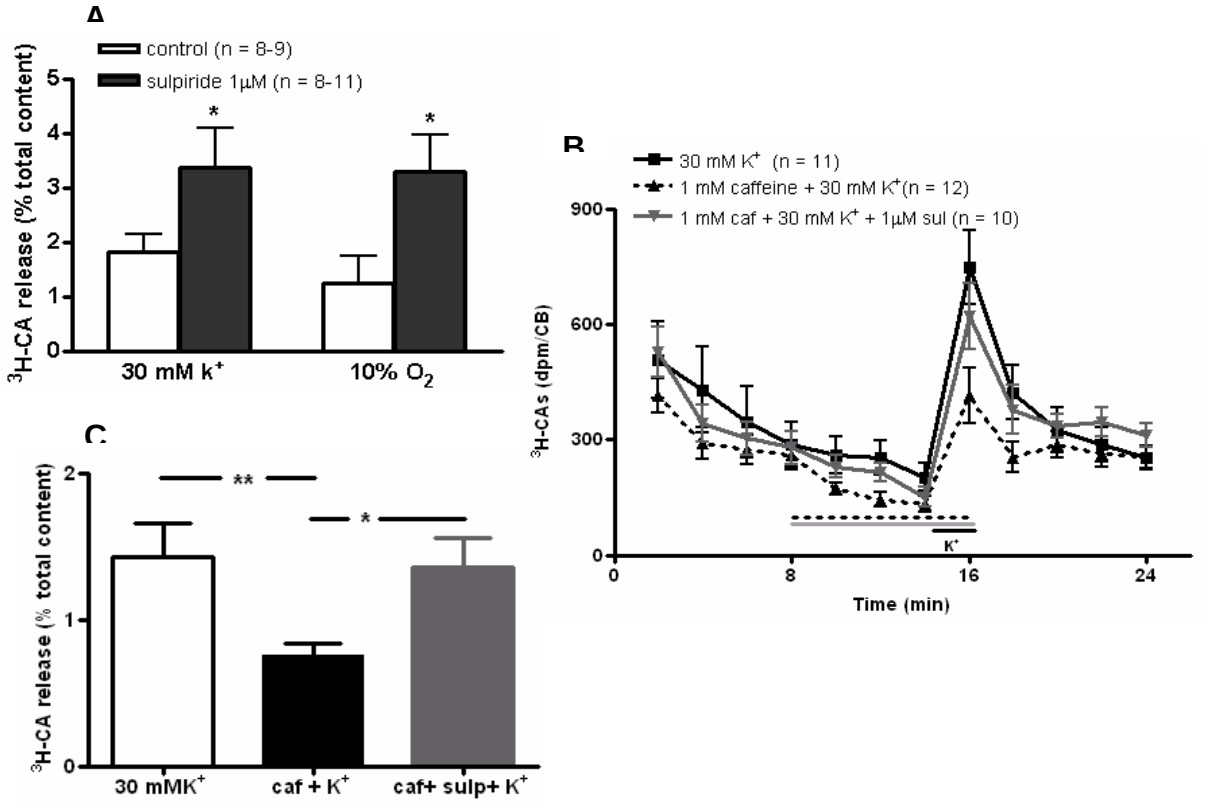


Figure 38 Effect of sulpiride on the release of ^{3}H-CA evoked by 30 mM extracellular K^+ and by 10% O_2 (**A**) and reversion of the inhibitory effect of caffeine on the release of ^{3}H-CA from rat carotid body (**B,C**). **A**. Sulpiride (1μM) was applied 10 min prior to and during the stimuli application, and potentiated the response elicited by both stimuli. 0% effect corresponds to 1.915 ± 0.293 and to 1.990 ± 0.277% of total ^{3}H-CA content respectively, in the absence and in the presence of sulpiride. **B**. Time course of the effect of sulpiride on the inhibitory effect of caffeine on the release of ^{3}H-CA evoked by 30 mM K+. Samples collected each 2 min. Caffeine and sulpiride were applied jointly at 10 min and 6 min before, during, and 2 min after the application of the stimuli. **C**. Sulpiride (SULP; 1 μM) almost completely reverts the inhibitory effect of 1 mM of caffeine (CAF) on the evoked release of ^{3}H-CAs. 0% effect corresponds to 0.382 ± 0.058 in the absence of drugs and 0.318 ± 0.094 in the presence of caffeine and 0.388 ± 0.172 % of total ^{3}H-CA content, in the presence of sulpiride and caffeine, respectively. * $P < 0.05$;** $P < 0.01$ One and Two-Way ANOVA with Bonferroni´s multi-comparison test. Vertical bars represent means± SEM

6.3.3. Effect of D_2 antagonists on the release of ^{3}H-CA release by rat carotid body and its potentiation with NECA

Since sulpiride did not modify the basal release of ^{3}H-CA, we tested other D_2 dopamine receptor antagonists, domperidone and haloperidol.

Domperidone (0.5 nM - 5 μM) and haloperidol (0.01-10 μM) increased the basal release of ^{3}H-CA from rat CBs in a concentration-dependent manner (Figure 39A). Maximal increases (E_{max}) of 123.5% and 122.3% on the basal release of ^{3}H-CA occurred with 5 μM of domperidone and 10 μM of haloperidol, respectively. The EC_{50} were 81 and 147 nM for domperidone and haloperidol, respectively. The effect of haloperidol was also tested on the release of ^{3}H-CA evoked by moderate hypoxia (10% O_2). As in normoxic conditions haloperidol increased the release of ^{3}H-CA in hypoxia in a concentration-dependent manner (Figure 39B) with a lower E_{max} (110.1%) and a higher EC_{50} (462 nM). The effect of NECA, an A_2 agonist, on the release of ^{3}H-CA produced by haloperidol both in normoxia and moderate hypoxia is shown in Figure 40.

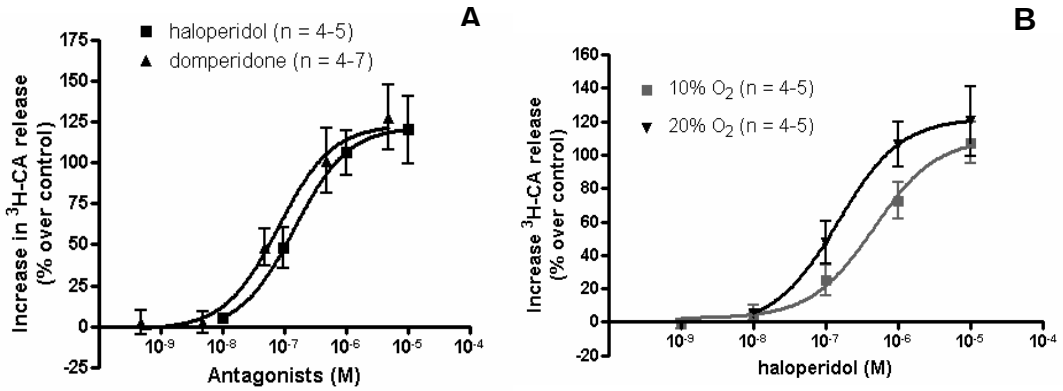


Figure 39 Dose-response curves for the effect of domperidone and haloperidol, D_2 dopamine antagonists, on the release of ^{3}H-CA from rat CB. **A**. Effect of domperidone and haloperidol in normoxic conditions. 0% effect in normoxic conditions corresponds to 0.927 ± 0.10% and to 1.010 ± 0.09% of the total ^{3}H-CA content for haloperidol and domperidone, respectively. **B.** Effect of haloperidol on the release of ^{3}H-CA in normoxic conditions and in response to moderate hypoxia (10% O_2). 0% effect for the effect of haloperidol in response to 10% O_2 corresponds to 5.563 ± 0.58% of the total ^{3}H-CA content. Values represent means ± S.E.M.

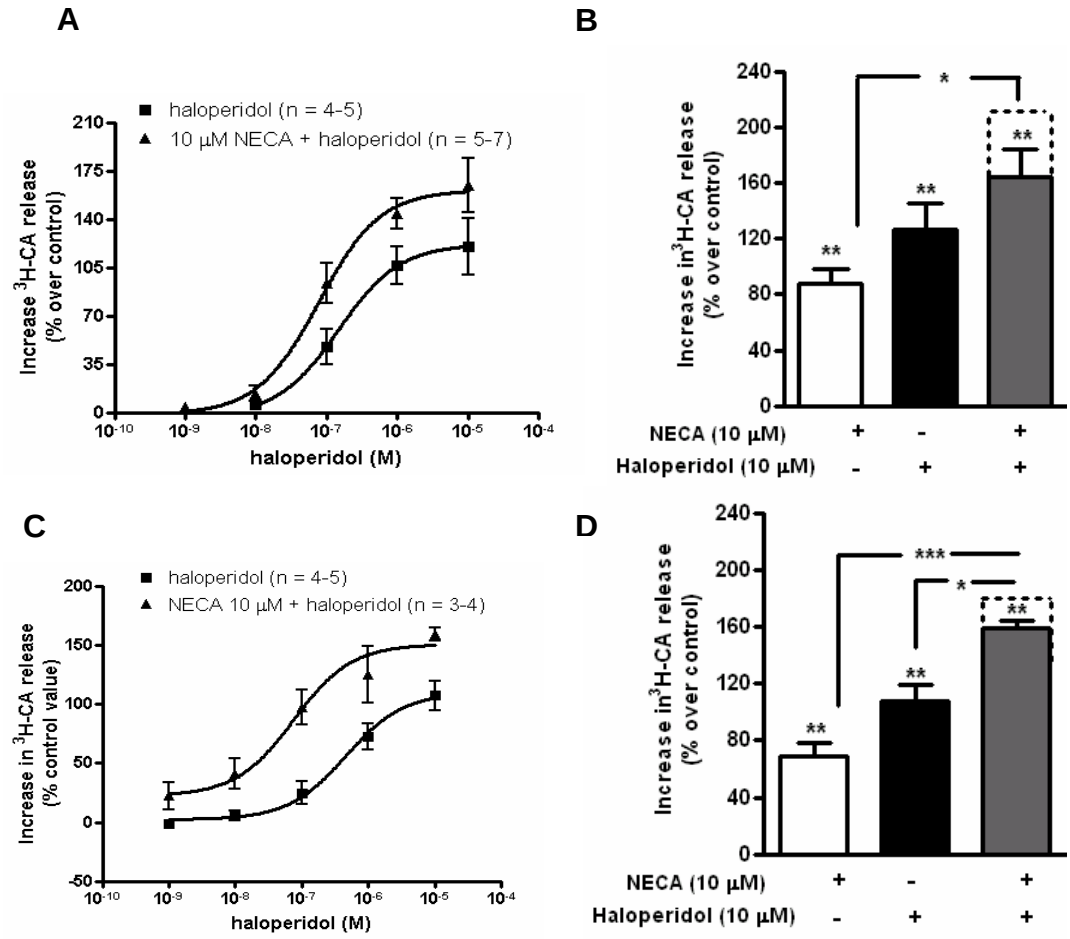


Figure 40 Potentiation of haloperidol, a D_2 dopamine receptor antagonist, effect on ^{3}H-CA release from CB by NECA, an A_2 adenosine receptor agonist in **(A, B)** normoxic conditions (20% O_2) and **(C,D)** in response to moderate hypoxia (10% O_2). In **B** and **C** the dotted lines represent the absolute values of the sum of the both drugs (NECA and haloperidol) applied separately in normoxia and hypoxia, respectively. Note that the effect of potentiation is synergistic and not additive. 0% effect corresponds to 0.927 ± 0.10% and to 5.563 ± 0.58% in normoxia and hypoxia, respectively. NECA was applied for 30 min between min 30 and 60 and haloperidol for 20 min between min 40 and 60 of the experimental protocol. Values represent means ± S.E.M.

It can be observed (Figure 40A and C) that the application of 10 μM (10 times the EC_{50} observed for the stimulation of ^{3}H-CA) of NECA moves the dose-response curve for the effect of haloperidol on basal and low intensity hypoxic release of ^{3}H-CA from the CB to the left. In Figure 40 B and D we can also observe that the potentiation of the effect of haloperidol by NECA in normoxia and moderate hypoxia is synergistic and not additive, since the effect of both drugs applied jointly is smaller than the sum of the effects of the drugs applied

separately. Maximal increases (E_{max}) and EC_{50} for the effect of haloperidol on the release of ^{3}H-CA from CB in normoxia and moderate hypoxia in the presence and absence of NECA are represented in Table 6.

Table 6 Effect of NECA (10 μM) on the efficacy and potency of haloperidol, a D_2 antagonist, in stimulating the release of CA from rat carotid body.

Physiological stimulus	Drugs	EC_{50}	Emax
20% O_2	Haloperidol	147 nM	122.3 ± 13.0
	NECA + Haloperidol	78 nM	161.5 ± 9.2
10% O_2	Haloperidol	462 nM	110.1 ± 10.8
	NECA + Haloperidol	79 nM	152.7 ± 12.7

NECA: 5'-(N-ethylcarboxamido)adenosine. Emax: maximal increase (%) in the release of ^{3}H-CA (mean ± SEM). EC_{50}: drug concentration that produced 50% of maximal effect. NECA by itself in this concentration caused 87.35 ± 10.73% increase in ^{3}H-CA (over control).

6.3.4. Inhibitory effect of the D_2 agonist, propylnorapomorphine, on the release of ^{3}H-CA from carotid body and its reversion by NECA

In order to investigate whether the interaction between D_2 and A_{2B} receptors that modulate the release of ^{3}H-CA in chemoreceptor cells would be antagonistic, we tested NECA on the effect of a D_2 agonist, propylnorapomorphine, on the release of this neurotransmitter from rat CB in normoxia and moderate hypoxia (see legend of Figure 41 for experimental protocol).

As expected, propylnorapomorphine (0.02-200 nM) inhibited the basal release of ^{3}H-CA (20% O_2) and the release in response to moderate hypoxia (10% O_2) in a concentration-dependent manner (Figure 41A). The maximal inhibitory effects (E_{max}) and IC_{50} of propylnorapomorphine in normoxia and moderate hypoxia are shown in Table 7.

To demonstrate the antagonistic interaction we applied 10 μM of NECA on the effect of propylnorapomorphine in normoxia and moderate hypoxia, and it was observed that NECA attenuated the inhibitory effect of the D_2 agonist on

the release of ^{3}H-CA from the CB in normoxia (Figure 41B) and in response to 10% O_2 (Figure 41C).

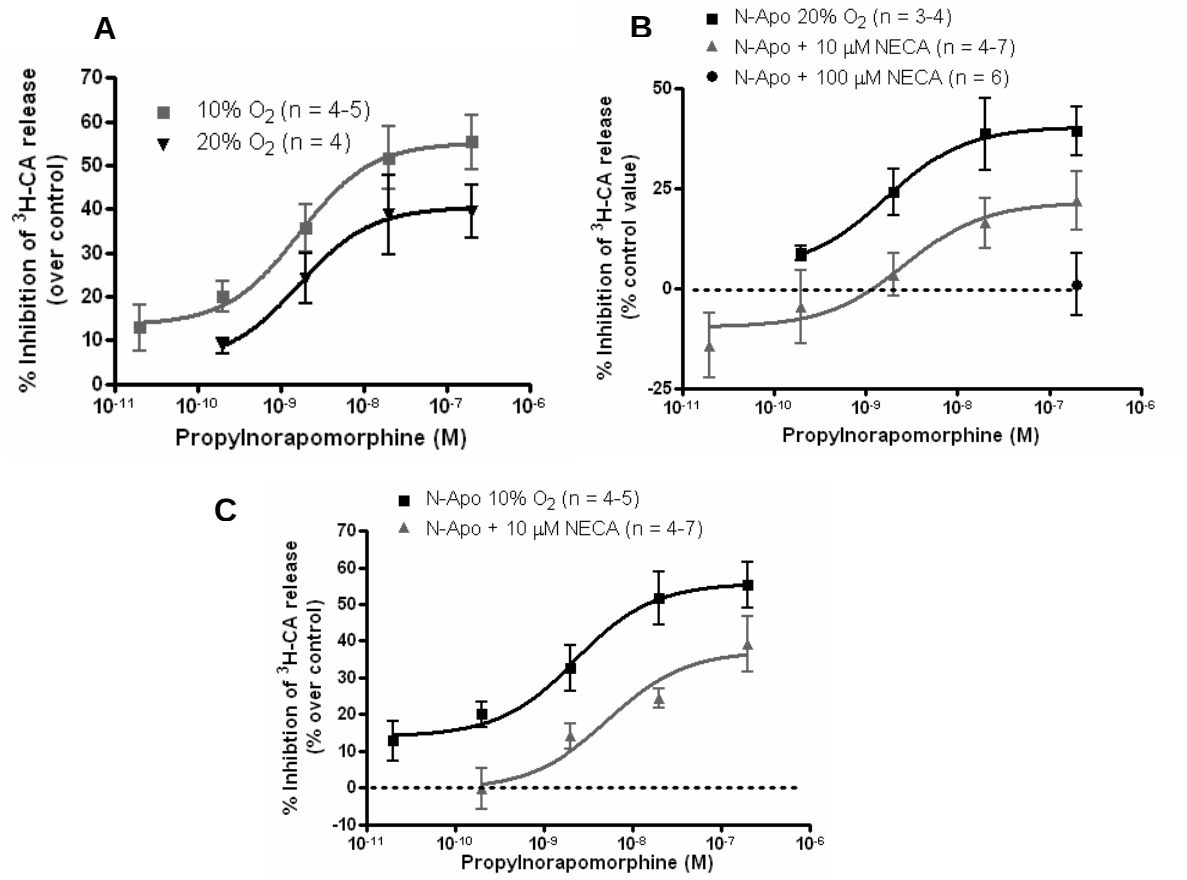


Figure 41 Effect of propylnorapomorphine (N-Apo) on the release of ^{3}H-CA from the carotid body in normoxic conditions and in response to moderate hypoxia (10% O_2) in the absence (A) and in the presence of NECA (B and C). **A**. Dose-response curves for N-Apo in normoxia and moderate hypoxia. P>0.05 **B**. Attenuation of the inhibitory effect of N-Apo by 10 µM of NECA, and abolishment of the inhibitory effect of N-Apo with 100 µM of NECA in normoxia. **C**. Attenuation of the inhibitory effect by 10 µM of NECA in response to moderate hypoxia. 0% effect corresponds to 1.716 ± 0.28% and to 4.231 ± 0.50 % of total ^{3}H-CA content in normoxia and hypoxia, respectively. Propylnorapomorphine was applied for 30 min between min 30 and 60 and NECA for 20 min between min 40 and 60 of the experimental protocol. Values represent means ± S.E.M.

It can also be observed that the inhibitory effect of 200 nM propylnorapomorphine on the basal release of ^{3}H-CA from chemoreceptor cells is completely abolished if a concentration of 100 µM of NECA is used (Figure 41B). Examining panels B and C of Figure 41, it appears that the attenuation of

the inhibitory effect of propylnorapomorphine by NECA is due to a displacement of the dose-response curves for the release of ^{3}H-CA to the right, both in normoxia and moderate hypoxia, suggesting that an antagonistic interaction between D_2 and A_{2B} receptors exists on chemoreceptor cells in the CB that control the basal release and low intensity stimulus evoked release of CA.

Table 7 Effect of NECA on the efficacy and potency of propylnorapomorphine, a D_2 agonist, in inhibiting the release of CA from rat carotid body.

Physiological stimulus	Drugs	IC_{50}	Emax
20% O_2	N-Apo	1.67 nM	40.50 ± 5.1
	10 μM NECA + N-Apo	2.69 nM	21.56 ± 6.2
10% O_2	N-Apo	2.32 nM	55.79 ± 4.7
	10 μM NECA + N-Apo	5.29 nM	37.2 ± 6.4

NECA: 5'-(N-ethylcarboxamido)adenosine. N-Apo: propylnorapomorphine. Emax: maximal % of inhibition over the control (mean ± SEM). IC_{50}: drug concentration that produced 50% of the maximal inhibition.

6.3.5. Effect of ionomycin on the release of ^{3}H-CA by carotid body modified by propylnorapomorphine and NECA

In the discussion of Chapter 5 we suggested that A_{2B} receptors operate at the CB in the last steps of the stimulus-secretion coupling process, since caffeine (and the other agents tested) produces identical effects in the response elicited by high K^+ (a non-specific depolarising stimulus) and hypoxia. In order to investigate at which step of the stimulus-secretion process the interactions between A_{2B} and D_2 are involved, we studied these antagonistic interactions on the release of ^{3}H-CA evoked by ionomycin. Ionomycin alters the permeability of cells, producing an increase in intracellular Ca^{2+} and, as a consequence, induces a Ca^{2+} dependent vesicular release of CA (for a review see Dedkova et al., 2000). This effect can be observed both in figure 42A on the time course of the ^{3}H-CA release (Figure 42A) as in the magnitude of the increase, in Figure 42B, where the application of 5 μM of ionomycin increased the release of ^{3}H-CA from rat CB by 8.78 ± 1.10%. The application of 200 nM of

propylnorapomorphine decreased the ^{3}H-CA release from CB evoked by ionomycin by 44% (Figure 42B).

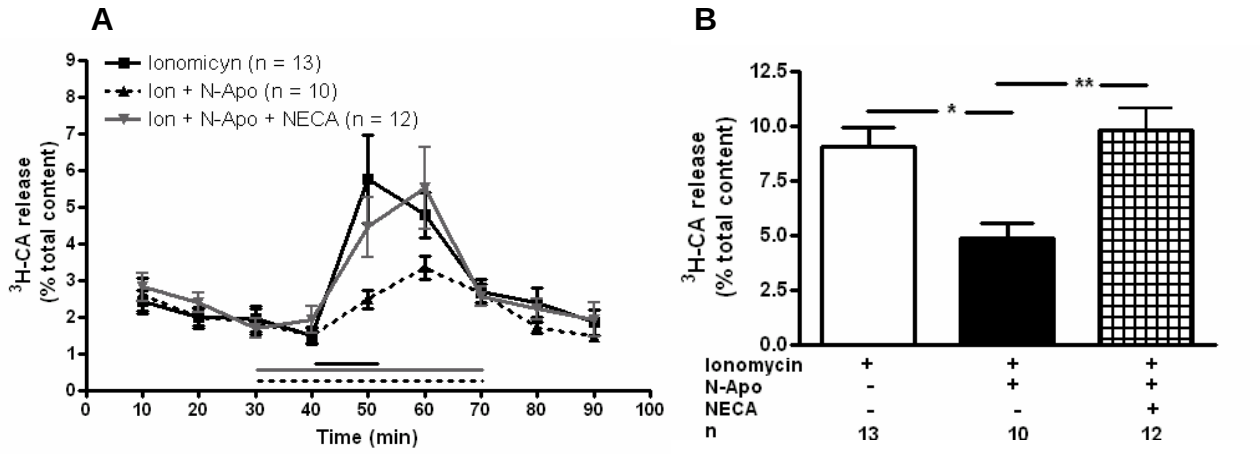


Figure 42 Inhibitory effect of propylnorapomorphine, a D_2 agonist, on the ionomycin evoked release of ^{3}H-CA from CB and reversion of the inhibitory effect by NECA. Panel **A** shows the time course for the effects of 200 nM propylnorapomorphine (N-Apo) and the reversion of this effect by 10 μM of NECA on the release of ^{3}H-CA evoked by 5 μM ionomycin. Ionomycin was applied for 10 min between min 40 and 50 of the experiment. N-Apo and NECA were applied 10 min prior and 20 min after stimulation with ionomycin. 0% corresponds to 1.946 ± 0.36, 1.49 ± 0.235 and 1.914 ± 0.36 % of ^{3}H-CA total content respectively for ionomycin in the absence and in the presence of N-APO and N-APO plus NECA. Panel **B** shows means ± S.E.M. of the effect of N-Apo in the absence and in the presence of NECA on ionomycin evoked release of ^{3}H-CA from CB. *P<0.05, **P<0.01; One-Way ANOVA with Dunnett's multi-comparison test.

When we tested the effect of NECA on the inhibitory effect of propylnorapomorphine on ^{3}H-CA release evoked by ionomycin we observed that the application of 10 μM of NECA completely reversed the inhibition (figure 43A and B). These results suggest that A_{2B} and D_2 receptors interact to modulate the release of ^{3}H-CA from rat CB between the steps of Ca^{2+} entry and increase in intracellular free Ca^{2+}, and the activation of exocytosis and neurotransmitter release, of the stimulus-secretion coupling process.

6.4. Discussion

These results provide pharmacological evidence that adenosine A_{2B} receptors and dopamine D_2 receptors, that are co-located in chemoreceptor

cells, interact in order to modulate the release of CA from rat carotid body chemoreceptor cells. In fact, we have found that antagonists of dopamine D_2 receptors facilitate the release of CA from rat chemoreceptor cells, this effect being prevented by antagonists of A_{2B} adenosine receptors and potentiated by agonists for the receptor. Furthermore, we have found that agonists of dopamine D_2 receptors inhibit the release of CA from rat chemoreceptor cells, this effect prevented by an agonist for A_{2B} receptors, suggesting the existence of an antagonistic receptor-receptor interaction between A_{2B} receptors and D_2 receptors in chemoreceptor cells.

Although our model used whole CBs, the results with the D_2 dopamine antagonists, sulpiride, haloperidol and domperidone, in normoxia, moderate hypoxia and in response to a moderate depolarising stimulus, are compatible with the presence of D_2 dopamine autoreceptors in the rat CB chemoreceptor cells, as previously described in the rabbit (Dinger et al., 1981; Fidone et al., 1991; Basson et al., 1997). These autoreceptors would control the release of CA from rat CB chemoreceptor cells by a negative feedback mechanism. Contrary to the effects of haloperidol and domperidone on the basal release of ^{3}H-CA, sulpiride did not modify release of CA in normoxia an effect that could be due to the action of this antagonist on other subtypes of dopamine receptors (Jaworski et al., 2001; Asaumi et al., 2006).

This study found some differences between the effects of D_2 receptor agonists and antagonists on the release of CA from CB chemoreceptor cells in normoxia and moderate hypoxia. The smaller effect of haloperidol on CA release from chemoreceptor cells in response to moderate hypoxia, in comparison with response to normoxia, can only be explained if haloperidol is not sufficient to antagonise all the D_2 receptors present in chemoreceptor cells and the endogenous dopamine released from chemoreceptor cells by moderate hypoxia can still exert some inhibition at D_2 receptors by the negative feedback mechanism diminishing the CA release. A contrary result, but in agreement with more pronounced inhibitory effects in stimulated preparations, was found for the effect of the D_2 agonist, propylnorapomorphine, on the release of CA in normoxia and moderate hypoxia. The more pronounced inhibitory effect of

propylnorapomorphine observed in moderate hypoxia, compared with normoxia, can be explained by the increased release of DA in response to hypoxia.

We would like to mention at the onset that throughout the discussion are going to attribute all the effects of NECA, which is an A_2 adenosine receptor agonist to an effect on A_{2B} adenosine receptors. In the previous (Figures 31, 32 and 33) and current (Figure 38) chapters of this thesis we have observed that NECA induces the release of ^{3}H-CA from CB. Since the effect is not mimicked by DPCPX in concentrations that affect A_1 receptors, or by CGS21680 and HE-NECA (A_{2A} selective agonists), we have concluded that A_{2B} adenosine receptors modulate the release of ^{3}H-CA from rat CB.

We have also found that the absence of effect of NECA under high intensity stimulation is in agreement with the previously described effect of caffeine on release of ^{3}H-CA from rat carotid body chemoreceptor cells in response to high intensity stimulus (Chapter 3, Section 5). Under high intensity stimulation conditions, adenosine would lose its effectiveness for two reasons: first, the release of adenosine itself tends to decrease with hypoxias of higher intensity, (Chapter 1, Section 3; Conde and Monteiro, 2006) and second, the high levels of DA in the surrounding milieu (released by a high intensity stimulus) would saturate D_2 receptors masking the effects of adenosine on D_2 receptors.

Receptor-receptor interactions can be synergistic and antagonistic in character and can be cooperative. When two different agonists to different receptors are combined they interact to enhance the efficacy of the combination (synergy) or to diminish the efficacy (antagonism). Alternatively, the agonist may not interact, and the effect of combination is obtained by adding the effects of each drug used individually (additivity) (Albritton et al., 2005; Fuxe et al. 2007). The fact that activation of D_2 receptors with propylnorapomorphine is diminished with increased concentrations of NECA, which activates A_{2B} receptors in the chemoreceptor cells in the CB (see Chapter 3, Section 5), demonstrates that we are in the presence of an antagonistic interaction between D_2 and A_{2B} receptors. With the results herein described and the protocols used in this work we cannot discern the location of the interaction, nor can we establish if the interaction is a functional coupling between A_{2B} and D_2

receptors, or at adenylyl cyclase level, or at both sites. Nevertheless, we can suggest that a functional coupling between A_{2B} and D_2 receptors in chemoreceptor cells could exist, as is described in the central nervous system (mostly in the striatum) between D_2 and A_{2A} receptors (Ferre et al., 1997; Hillion et al., 2002; Canals et al., 2003).

Dopamine D_2 receptors are expressed in chemoreceptor cells and co-localized with TH mRNAs in the same cells of the CB (Gauda et al., 1996). We found that A_{2B} adenosine receptors are also co-localized with TH in chemoreceptor cells (Conde et al., 2006) meaning that D_2 dopamine receptors and A_{2B} receptors are co-located in chemoreceptor cells and can interact to modulate the release of CA.

The interactions previously described between dopamine and adenosine in the CB observed when monitoring CSN activity in the cat (Ribeiro and McQueen, 1983) and ventilation in the rat (Monteiro and Ribeiro, 2000) have defined D_2 as the dopamine receptor involved, but could not identify the subtype of A_2 adenosine receptor participating in the interaction (see Introduction, Section 1.5.2.3.). Our data indicate that the subtype of adenosine receptor is the A_{2B} adenosine receptor. We cannot exclude the possibility that interactions between D_2 and A_{2A} receptors also exist in the CB. Nevertheless, the interaction herein observed is in agreement with the potentiation of the inhibitory effect of dopamine by adenosine observed in ventilation in the rat (Monteiro and Ribeiro, 2000).

In the current series of experiments, we have not specifically examined the mechanism of interaction, nonetheless some conclusions can be drawn and we believe that the model presented in Figure 43 reasonably fits our observations. D_2 autoreceptors in chemoreceptor cells form a negative feedback controlling the release of CA from chemoreceptor cells in the rabbit (Fidone et al., 1991; Basson et al., 1997) and in the rat (current results, Figures 37A and 38). A_{2B} adenosine receptors should be coupled to D_2 receptors, this coupling resulting in a decrease in the affinity of D_2 receptors for DA; through this coupling, endogenously released adenosine would be tonically activating the release of CA from chemoreceptor cells as it hinders the inhibitory action of DA. On the inhibition of A_{2B} receptors with caffeine, full inhibitory power of D_2 receptors appears, and thereby the decrease in CA release. Indeed, the

activation of A_{2B} adenosine receptors with NECA decreases the affinity of the D_2 receptor agonist, propylnorapomorphine in CB chemoreceptor cells. Under intense stimulation, adenosine would lose its effectiveness due to the two facts cited above.

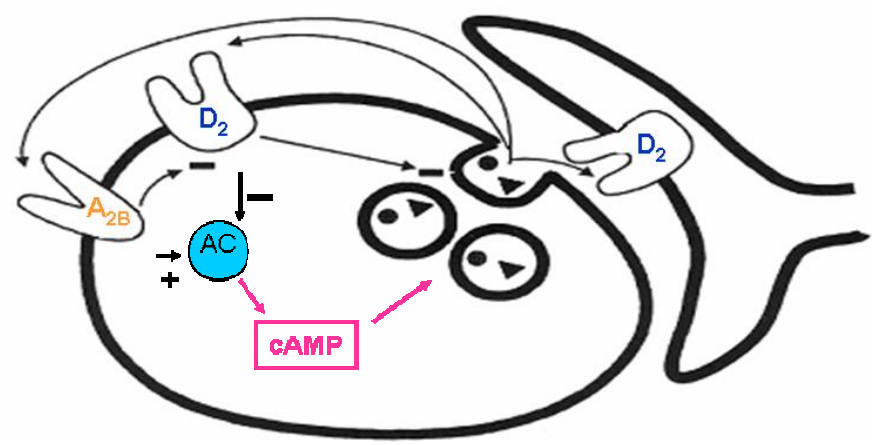


Figure 43 Mechanisms postulated for the interaction between A_{2B} and D_2 receptors in chemoreceptor cells that modulate the release of CA from rat carotid body (see text). AC – adenylyl cyclase; cAMP - 3',5'-adenosine monophosphate

We cannot exclude an interaction at the adenylyl cyclase level, since both the receptors exert opposite actions on adenylyl cyclase and therefore on cAMP levels (Figure 43). In fact, the potentiation of the effect of haloperidol on the release of CA from chemoreceptor cells by NECA (Figure 39), the combined effect being smaller than the sum of effects of the two drugs applied separately, suggests that in part (but not totally) the interaction can occur at this level. Indeed, in some preparations, it is described that this type of interaction between receptor-receptor and at second messenger level can co-exist, like in striatal slices *in vitro* (Svenningsson et al., 1998) and in cells of Chinese hamster ovary (Kull et al., 1999). However, experiments to measure cAMP levels in the CB in order to clarify if interactions at second messenger level exist would require additional work. To investigate and confirm the existence of an antagonistic interaction between A_{2B} receptor and D_2 receptor in the plasmatic membrane of chemoreceptor cells in the CB, experimental studies for analysing receptor collocation and co-trafficking, including co-clustering and co-internalisation, with computer assisted image analysis are needed. Important

methodologies for analysis of collocation and co-trafficking of receptors would be atomic force or confocal microscopy.

This type of coupling between autoreceptors and A_{2B} receptors is not unique to chemoreceptor cells. Talaia et al. (2005) found that adenosine acting via A_{2B} receptors augments the release of noradrenaline from the sympathetic endings innervating the vas deferens, this effect being abolished by yohimbine, a blocker of presynaptic α2-adrenoreceptors. This type of interaction, in which A_{2B} receptors control the release of dopamine, was previously observed in the rat striatum by Okada et al. (1996). They observed that CGS 21680 did not affect striatal extracellular dopamine levels, but stimulatory and inhibitory effects of the A_{2A} agonist and antagonist, DMPA and DMPX, respectively, were observed when A_1 receptors were blocked, suggesting that A_{2B} receptors modulate the release of dopamine in the striatum under A_1 blockade.

We have discussed, and previously suggested (Chapter 3, Section 5) that A_{2B} receptors are coupled towards the last steps in the stimulus-secretion coupling process, since caffeine and the drugs that mimic its effects have produced similar effects on the response elicited by high K^+ (a non-specific depolarising stimulus) and by hypoxia. In an attempt to discover at which step of the stimulus-secretion process the interactions between A_{2B} and D_2 receptors are involved, we have studied this interaction on the release of ^{3}H-CA evoked by ionomycin. Ionomycin is a calcium ionophore, and it is generally assumed that these substances directly facilitate the transport of Ca^{2+} across the plasma membrane. The Ca^{2+} signal induced by the Ca^{2+} ionophores, and in this case by ionomycin consists of three components: the first component is due to the activation of Ca^{2+} influx through native Ca^{2+} channels and is sensitive to drugs which inhibit the receptor-operated Ca^{2+} influx; the second component originates from phospholipase C-dependent mobilisation of Ca^{2+} from intracellular stores; and the third ionophoric component is very small at low concentrations of the ionophores (Himmel et al., 1990; Dedkova et al., 2000). The Ca^{2+} signal induced by ionomycin that produces an increase in Ca^{2+} intracellular concentrations, originating the release of neurotransmitters eliminates an effect of the interaction between D_2 and A_{2A} receptors on the

entire hypoxic transduction cascade until step 6 in our study. The fact that NECA abolishes the inhibitory effect of propylnorapomorphine on the ionomycin evoked release of ^{3}H-CA supports the above suggestion and indicates that the interaction between the A_{2B} and D_2 receptors present in CB chemoreceptor cells modulating CA release is between the step 6 and step 7 of the stimulus-secretion coupling process (see Figure 4 or Chapter 3 discussion for transduction cascade of hypoxic signalling). Nevertheless, clarification of the intimate mechanisms of interaction and action on the exocytotic process would require additional experiments.

To summarise, we can assume that an antagonistic interaction between A_{2B} and D_2 receptors exists in the CB that modulates the release of CA from chemoreceptor cells.

7 – CHAPTER 5 – EFFECT OF CHRONIC CAFFEINE INTAKE ON THE CAROTID BODY CHEMOSENSORY ACTIVITY IN CONTROL AND CHRONIC HYPOXIC RATS

7.1. Introduction and aim

Acute administration of caffeine caused an inhibition in carotid body chemoreceptor activity due to the antagonism of adenosine A_{2A} and A_{2B} receptors (see Chapter 3, Section 5). The absence of data on the effects of chronic caffeine on CB function prompted us to study the effects of chronic caffeine consumption and therefore the permanent blockage of adenosine receptors on the peripheral control of breathing and on ventilation. We also aimed to investigate the role of adenosine in chronic hypoxia, through the permanent blockade of adenosine receptors (chronic caffeine intake).

To study the effects of chronic caffeine intake on carotid body chemosensory activity in control and chronically hypoxic animals we have: assessed the function of chemoreceptor cells by measuring the content, synthesis and release of catecholamines and the expression of TH as well as the release of adenosine and ATP; determined the functional activity of the intact organ by measuring the chemosensory activity in the CSN in normoxia, and acute hypoxia and hypercapnia; and assessed the global effect measuring the ventilatory responses to acute hypoxia and hypercapnia. In some cases SCG were used as control preparation to assess the specificity of the effects of chronic hypoxia, chronic caffeine intake and both in CB chemoreceptor function.

7.2. Materials and methods

7.2.1. Chronic caffeine intake in control and chronic hypoxic animals

The experiments were performed in Wistar rats of both sexes aged 3 months (200-350g) obtained from vivarium of the Faculty of Medicine of the University of Valladolid. The control animals were maintained at a normal atmosphere (room air: 20 - 21% O_2). With respect to the chronically hypoxic rats, they were kept for 15 days in a chamber equilibrated with a gas mixture of 11 -12% O_2 in 88-89 % N_2; $PO_2 \approx 90$ mmHg N_2 (hypoxic atmosphere) with a flow of 3L/min (Figure 44). To study the chronic caffeine intake we subdivided the

groups of controls and hypoxic rats into two (with and without caffeine), and kept the rats drinking 1g/L of caffeine for 15 days (Figure 44), according to the protocols commonly used to chronically treat animals with caffeine (Gasior et al., 2002; da Silva et al., 2003; Karcz-Kubicha et al., 2003).

Another group of control and chronically hypoxic animals treated with caffeine for 8 days only were inserted into the study in order to investigate the effect of caffeine and the significance of adenosine on ventilation during a short period of chronic hypoxia. The study design, including the definition of the groups, was equal to that represented in Figure 44 but for only 8 days.

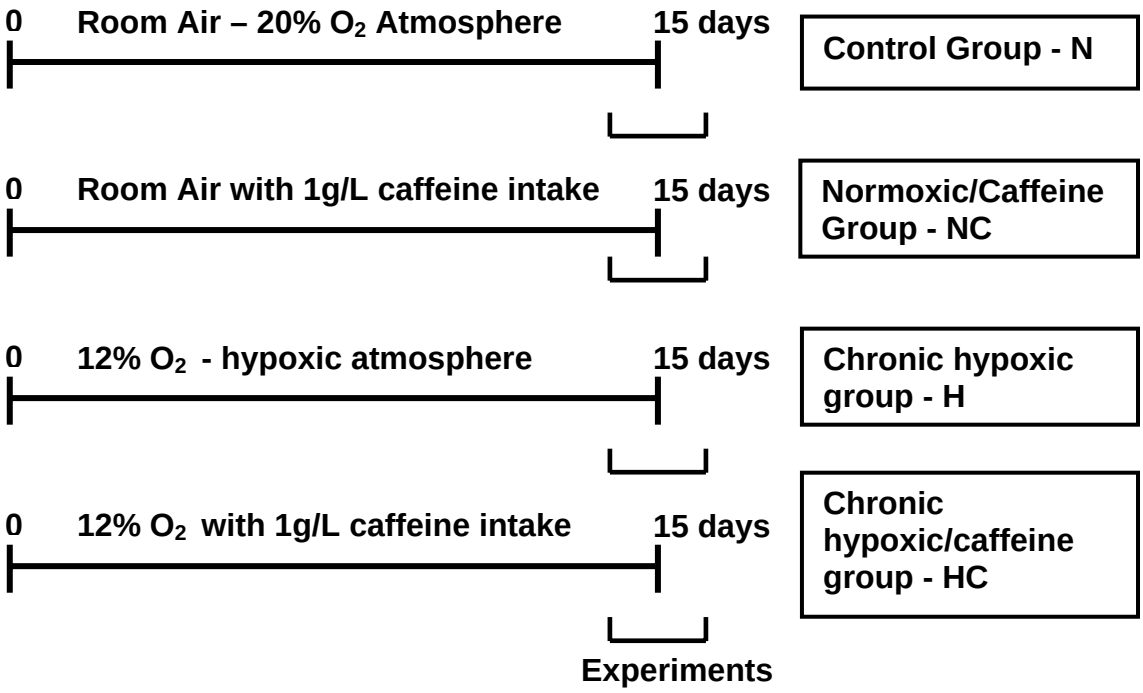


Figure 44 Experimental design with time courses and the paradigms to which rats were submitted, and the definition applied to the distinct groups of animals.

The accumulation of CO_2 and water vapour inside the chamber was avoided by air renewal and by the presence on the ground of the chamber of a layer of soda lime granules. Each 4 days the chamber was opened to be cleaned and restocked with water and food. After each cleaning cycle, a high flow of gas was employed in order to drop quickly the PO_2 in the chamber. The temperature and PO_2 in the chamber were monitored with a humidity thermometer and by an oximeter, respectively, and the animals were always

kept with a seasonal day/light rhythm, with temperature controlled between 23-26ºC. The Institutional Committee of the University of Valladolid for Animal Care and Use approved the protocols.

For the experiments aimed to measure endogenous CA content and synthesis of ^{3}H-CA we used 4-8 CBs/experiment and 4-8 SCGs/experiment. In the experiments where the release of endogenous ATP, adenosine and CA were analysed, 4 CBs were used in each experiment, due to the small dimensions of the CB (≈50 µg, Conde et al., 2006a) and the limit of detection of the techniques used. The CBs and SCGs were cleaned of nearby connective tissue following procedures previously described (Vicario et al., 2000a, Chapter 3, Section 5). For the recording of CSN activity we followed the procedure described in Section 5.2.3.. In all instances animals were killed by an intracardiac overdose of sodium pentobarbital.

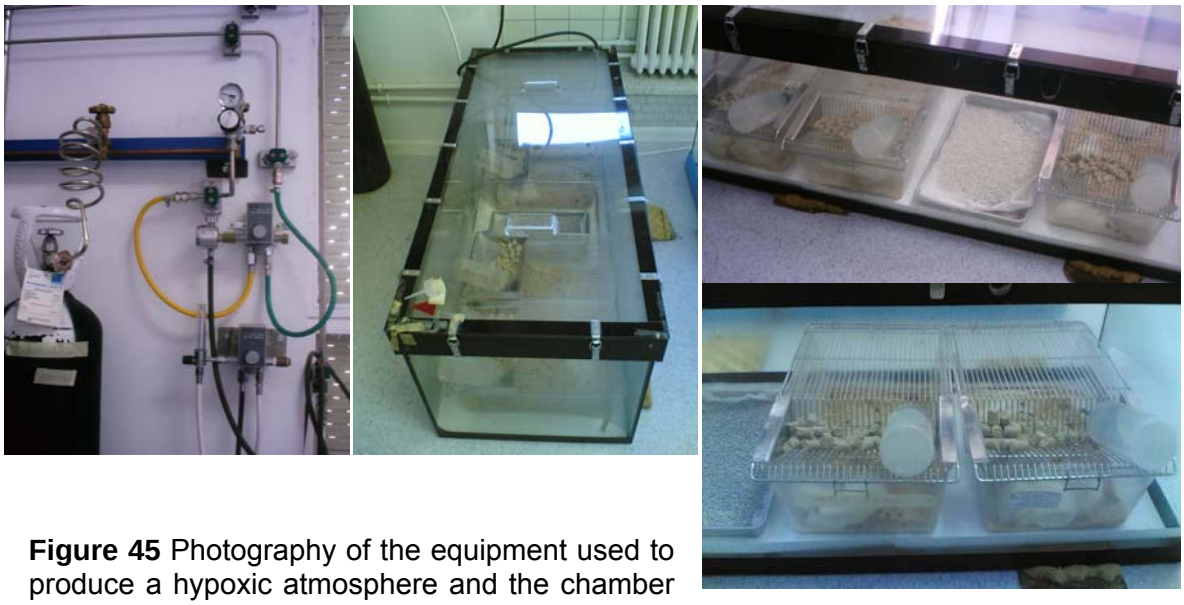


Figure 45 Photography of the equipment used to produce a hypoxic atmosphere and the chamber into which animals were inserted to be submitted to chronic hypoxia.

7.2.2. Measurement of tissue catecholamines content

CBs and SCG were cleaned and transferred to cold Eppendorf tubes containing 80 µl and 200 µl of 0.4 N perchloric acid, respectively, and after a few minutes in acid, the CBs and SCGs were weighed using an electrobalance (Supermicro, Sartorius), and glass-to-glass homogenized at 0-4 ºC. The homogenates of CBs and SCGs plus an aliquot of 40 µl and 100 µl,

respectively, of 0.4 N perchloric acid, used to quantitatively collect the tissue homogenate, were centrifuged (10 min; 0-4 ºC) in a microfuge (Beckmann, Madrid). The CA content in the tissues was quantified by HPLC as described in Section 7.2.3.

7.2.3. Labelling of CA stores to measure the rate of synthesis of ^{3}H-CA

The stores of CA in the CB and in SCG were labelled by incubating the organs in a solution containing tyrosine, the natural precursor of CA, labelled with tritium (3,5-^{3}H-tyrosine, Amersham). 4 to 8 CBs were placed in small glass vials containing 0.5 ml of Tyrode solution and placed in a shaker bath at 37 ºC. The concentration of ^{3}H-tyrosine was 30 µM and its specific activity was 6 Ci/mmol. In addition, the incubating solution contained 100 µM 6-methyl-tetrahydropterine and 1 mM ascorbic acid, cofactors for tyrosine hydroxylase and dopamine-β-hydroxylase, respectively (Fidone & Gonzalez, 1982). Incubation lasted 2h.

On completion of the labelling period, the CBs and SCG were transferred to vials containing 10 ml of ice-cold precursor-free Tyrode solution to wash out the ^{3}H-tyrosine present in the extracellular space for a period of 5 min. Afterwards the CBs and SCG were transferred to cold Eppendorf tubes containing 80 µl and 200 µl of 0.4 N perchloric acid, respectively, and after a few minutes in acid, the CBs and SCGs were weighed using an electrobalance (Supermicro, Sartorius), and glass-to-glass homogenized at 0-4 ºC. The homogenates of CBs and SCGs plus an aliquot of 40 µl and 100 µl, respectively, of 0.4 N perchloric acid, used to quantitatively collect the tissue homogenate, were centrifuged (10 min; 0-4 ºC) in a microfuge (Beckmann, Madrid). ^{3}H-CA synthesised and free ^{3}H-tyrosine present in the tissues were measured in the supernatants, and ^{3}H-tyrosine incorporated into proteins was measured in the pellets.

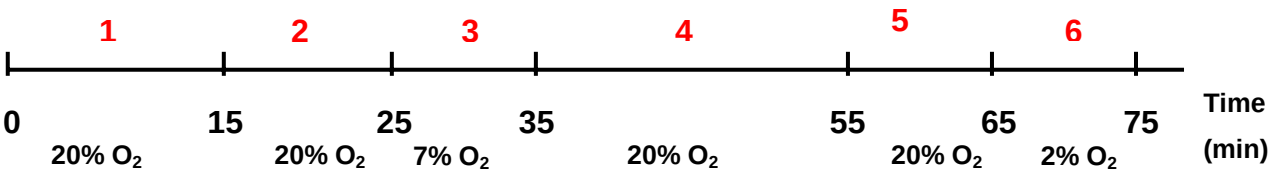
^{3}H-tyrosine incorporated into proteins was determined by liquid scintillation spectrometry of the pellets resulting from centrifugation of the acid homogenates of the CB and SCG. Free ^{3}H-tyrosine accumulated by the CBs and SCGs during the period of synthesis was also measured by liquid scintillation counting: an aliquot of the supernatants was counted and the

radioactivity present in it minus radioactivity in the form of ^{3}H-CA was taken as free intracellular ^{3}H-tyrosine.

For the analysis of endogenous unlabelled and ^{3}H-labeled CA in the tissue extracts and for the analysis of the endogenous release of CA, aliquots (10-100 µl) of supernatants were directly injected into an HPLC system composed of a Milton Roy CM 400 pump, a Waters C18 (particle size 4 µm) column, a Waters U6K injector, a Bioanalytical Systems LC-4A electrochemical detector (set at a holding potential of 0.75 mV and a sensitivity of 1 to 5 nA). The signal coming out of the detector was fed to an analogue to digital converter controlled by Peak Sample Chromatography System Software (Buck Scientific, East Northwalk, CT, USA). Identification and quantification of endogenous CA in tissue samples were done against external standards. In the case of the synthesis experiments, identification of ^{3}H-catechols was similarly done against external standards, but quantification was made by collection of the HPLC column effluents correspondent to the peaks of interest and scintillation counting of the collected effluents.

7.2.4. Endogenous release of ATP, adenosine and CA from carotid body

The CBs were incubated in 500 µl in Tyrode containing EHNA (25 µM), an inhibitor of adenosine deaminase, tyrosine (20 µM), ascorbic acid (1 mM) and 6-methyl-tetrahydropterine (100 µM), the last two substances being cofactors of dopamine-β-hydroxylase and tyrosine hydroxylase, respectively. The incubation mediums were kept at 37ºC and continuously bubbled with 20% O_2/5% CO_2/75% N_2 saturated with water vapour, except when hypoxic stimuli were applied. The solutions were renewed at each fixed time, as described above in the protocol, and all fractions collected except in the 1st and 4th period.



The collected fractions were acidified with 300 µl of PCA 3M. At the end of the experiment the CBs were immersed in 100 µl of PCA 3M and weighed. The collected fractions were kept for 10 min at 0 ºC and then centrifuged at 12000 g for 10 min (4 ºC). The supernatant was recovered and 200 µl were reserved to quantify CA. The nucleotides (ATP and adenosine) were also extracted from the supernatant as described in Section 3.2.6.. Endogenous CA release was quantified by HPLC with electrochemical detection (Section 7.2.4), adenosine by HPLC with UV detection (Section 3.2.7) and ATP by a bioluminescence luciferin-luciferase assay (Section 7.2.5.).

7.2.5. Quantification of ATP by bioluminescence luciferin-luciferase assay

For ATP quantification 100 µL of the samples were added to 100 µL of luciferin-luciferase (FLE50, Sigma) and to 2 mL of buffer (in mM: HEPES 20; $MgCl_2$ 25; Na_2HPO_4 5). The reaction begins when the enzyme is added to the mixture. The samples were analysed in triplicate for 1 minute by bioluminescence using a luminescence counter (Beckham). Quantification of endogenous ATP released by CB was done against external standards.

7.2.6. Western blot analysis of tyrosine hydroxylase expression in the carotid body

CBs and SCGs were homogenized in Zurich medium (Tris-HCl 10 mM; EDTA 1 mM; NaCl 150 mM; Triton X-100 1%; Sodium cholate 1%; SDS 0.1%) and a cocktail of protease inhibitors. Protein concentrations of the homogenates were measured by a Micro-BCA protein Assay (Pierce, Spain). Samples of the homogenates and the pre-stained molecular weight markers (Precision, BioRad, Spain) were separated by SDS-Page (10% with a 5% concentrating gel) under reducing conditions and electro-transferred to polyvinylidene difluoride membranes (0.45 µM, Millipore, Spain). After blocking for 1 hour at room temperature with 5% milk in Tris-buffer saline, pH 7.4 containing 0.01% Tween 20 (TTBS) (BioRad, Spain), the membranes were incubated overnight at 4 ºC with a mouse anti-TH (1:20000, Abcam, Cambridge, UK). After 3 washing periods of 10 min with TTBS the membranes were incubated with anti-mouse

HRP (1:10000, Sta Cruz Biotechnology, USA) in TTBS for 1 hour at room temperature. After 3 washes of 10 min with TTBS the membranes were incubated with enhanced chemiluminescence **(**ECL, Sta Cruz Biotechnology, USA). The density of the bands on Western blots was quantified by a PDI Scanner and by Origin 7.0.

The membranes were then re-probed and tested for ß-actin immunoreactivity (bands in the ≈ 45 kDa region) in order to compare and normalise the expression of TH with the amount of protein loaded. Briefly, the membranes were washed several times with TTBS, and then incubated for 2 hours at room temperature with anti-mouse ß-actin (1:5000, Sigma, Madrid, Spain). After 3 washing periods of 10 min with TTBS the membranes were incubated with anti-mouse HRP (1:10000, Sta Cruz Biotechnology, USA) as previously described.

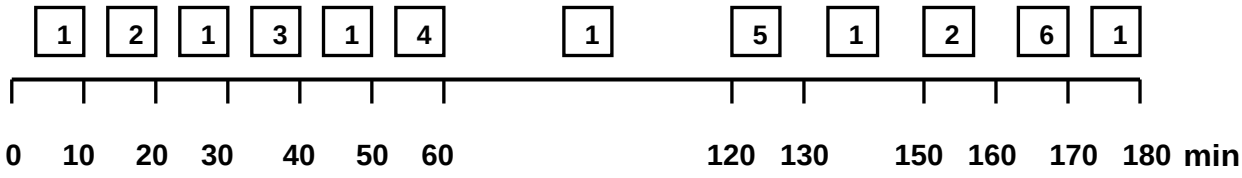
7.2.7. Recording of carotid sinus nerve activity

The CB-CSN preparation was transferred to a recording chamber mounted on a dissection microscope (Nikon) and superfused (37 ºC) with bicarbonate/CO_2 buffered saline (in mM: NaCl 120; $NaHCO_3$ 24; KCl 3; $CaCl_2$ 2; $MgCl_2$ 1.1; glucose 5; pH 7.40). Recordings of single or few fiberfibres of CSN were made as described in section Section 5.2.3.. The CSN chemosensory activities in response to hypoxic and hypercapnic stimulation were achieved by perfusing the preparations with solutions equilibrated with gas mixtures containing 0% or 5% O_2 + 5% CO_2 + balanced N_2 and 20% O_2 + 20% CO_2 + balance of N_2, respectively.

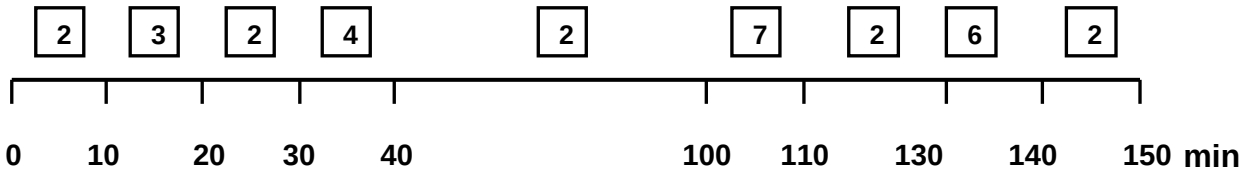
7.2.8. Whole-body plethysmographic recordings of ventilatory responses in response to hypoxia and hypercapnia

Ventilation was measured in conscious freely moving rats by whole body plethysmography. The system (Emka Technologies, Paris, France) consisted of 5-litre methacrylate chambers continuously fluxed (2 l/min) with controlled temperatures within the thermo-neutral range (22-24ºC). Tidal volume (V_T; ml) respiratory frequency (f; breaths/min (bpm)) and minute ventilation (V_E;

ml/min/Kg) were measured. Briefly, the rats were placed in the plethysmographic chamber and breathed room air for at least 30 min until they adapted to the chamber ambient and acquired a standard resting behaviour. Thereafter we started recording the ventilatory parameters according to the protocols used. For normoxic rats the protocol consisted of:



For chronic hypoxic animals the protocol consisted of:



The numbers correspond to: **1) 20 % O_2; 2) 12% O_2; 3) 10% O_2; 4) 7% O_2; 5) 20% O_2 + 5% CO_2; 6) 100% O_2; 7) 12 % O_2 + 5% CO_2.** All the gases were balanced with N_2 and applied at a flow of 2 l/min.

The pressure change within the chamber reflecting tidal volume (V_T) was measured with a high-gain differential pressure transducer. Ideally the frequency of pressure fluctuations is identical to breathing movements; spurious fluctuations of the pressure due to animal movements were electronically rejected. The amplitude of the pressure oscillations is proportionally related to V_T; a calibration of the system by injections of 0.2 to 0.5 ml air into the chamber allowed a direct estimation of V_T. Pressure signals were fed to a computer for visualisation and storage for later analysis with EMKA software.

7.2.9. Drugs and chemicals

6-methyl-tetrahydropterine, ascorbic acid, adenosine, ATP, caffeine and dopamine were obtained from Sigma (Sigma-Aldrich, Madrid, Spain). ^{3}H-tyrosine was obtained from Amersham (Madrid, Spain). Adenosine, ATP and dopamine were prepared as 5 mM stock solutions in water. The monoclonal tyrosine hydroxylase antibody was purchased from Abcam (Cambridge, UK) and the mouse antibody ß-actin from Sigma (Madrid, Spain). The horseradish peroxidase (HRP)-conjugated anti-mouse IgG was purchased from Santa Cruz Biotechnol (USA).

7.2.10. Data analysis

The amount of CA, ATP and adenosine endogenously released from CBs was expressed in pmol/mg tissue after the division of the absolute values obtained by four (four CBs used in each incubation) and corrected for the volume used and the medium weight of the 4 CBs. Data were evaluated using Graph Pad Prism Software, version 4 and were presented as mean ± SEM. The significance of the differences between the means was calculated by One and Two-Way Analysis of Variance (ANOVA) with Dunnett's and Bonferroni multiple comparison tests, respectively. *P* values of 0.05 or less were considered to represent significant differences. The density of the bands on Western blots was quantified by a PDI Scanner and by Origin 7.0.

7.3. Results

There was a stable intake of caffeine throughout the experiment in rats chronically exposed to caffeine and chronically exposed to both caffeine and hypoxia. The caffeine was added in the drinking water at a concentration of 1 mg/ml. The daily caffeine intake was 82.01 ± 6.34 (mg/rat/day) in rats exposed for 2 weeks to normoxia with chronic caffeine ingestion, and 79.37 ± 2.04 (mg/rat/day) in rats chronically exposed for 2 weeks to caffeine and hypoxia. Water consumption in control groups was similar to caffeine-drinking rats and the rats presented comparable weights. These values of caffeine are

comparable to those described for caffeine consumption in rats with similar chronic caffeine treatment (Gasior et al., 2002; da Silva et al., 2003; Karcz-Kubicha et al., 2003).

At the outset of the results we would like to mention that in normoxic rats all the experimental basal levels were considered levels in response to 20% O_2. For chronically hypoxic rats we performed all the control experiments (basal levels) in 12% O_2, since the animals were maintained for 8 (for plethysmographic recordings) or 15 days in an atmosphere of 12% O_2. The gases were mixed with 5% CO_2 if the experiments were performed *in vitro*.

We would like to mention that, in an attempt to explain some of the differences between the results in chronic hypoxia and chronic hypoxia plus chronic caffeine in the CA dynamics, and since the weights of the CBs in these conditions vary so much, we have also expressed all graphs as pmol/CB obtaining the same profiles of variation with the same significances. We chose to represent all graphs as pmol/mg tissue.

7.3.1. Effect of chronic caffeine intake on carotid body and superior cervical ganglion weight in control and chronic hypoxic rats

Figure 46A shows the effect of chronic hypoxia, chronic caffeine intake, and the effect of caffeine intake on rats submitted to chronic hypoxia, on the CB weight. In 16 control CBs (normoxic animals) the mean CB weight was 49.94 ± 2.95 µg, this value being very similar to that previously described by Conde et al. (2006a). The value found for control CBs was not different to that of normoxic rats submitted to chronic caffeine treatment (50.07 ± 4.81 µg; n = 15). Chronic hypoxia doubled the weight of rat CB, reaching weights of 98.79 ± 4.55 µg (n= 14). Interestingly, the animals that were submitted to both treatments, chronic hypoxia and chronic caffeine intake, presented CB weights smaller than the chronically hypoxic ones, 67.80 ± 5.01 µg (n= 15), suggesting that caffeine can reverse or prevent the enlargement of CB produced by chronic hypoxia. In order to investigate if these modifications produced by chronic hypoxia and by chronic hypoxia applied conjunctly with chronic caffeine intake were specific of the CB, we also weighed the SCGs of the same animals, and we observed that

none of the treatments, nor the application of both conjunctly modified the weight of SCGs (Figure 46B).

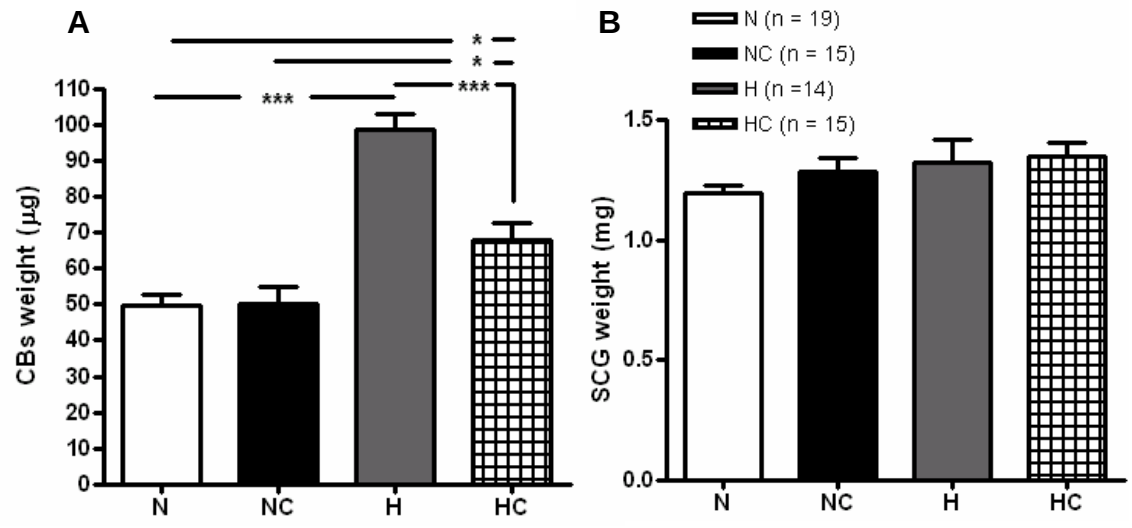


Figure 46 Effect of chronic caffeine intake, chronic hypoxia and both treatments jointly applied, on the weight of CBs **(A)** and SCGs **(B)**. Data represent means ± SEM. *P<0.05;***P<0.001; One - Way ANOVA with Bonferroni multi-comparison test. N – normoxic rats, NC - normoxic submitted to chronic caffeine intake, H – chronic hypoxic rats, HC – chronic hypoxic rats submitted to chronic caffeine intake.

7.3.2. Effect of chronic caffeine intake on CA content of the carotid body in control and chronic hypoxic rats

Figure 47 shows the mean content of CA, DA and norepinephrine (NE), in CB of the rats of the different groups; data are expressed as pmol/mg tissue. Mean DA content in control CB (Group N) was 311.1 ± 57.60 pmol/mg tissue (n = 10) (Figure 47A). Chronic caffeine intake in normoxic rats did not modify the DA content (322.3 ± 32.76 pmol/mg tissue, n = 8) in the CB.

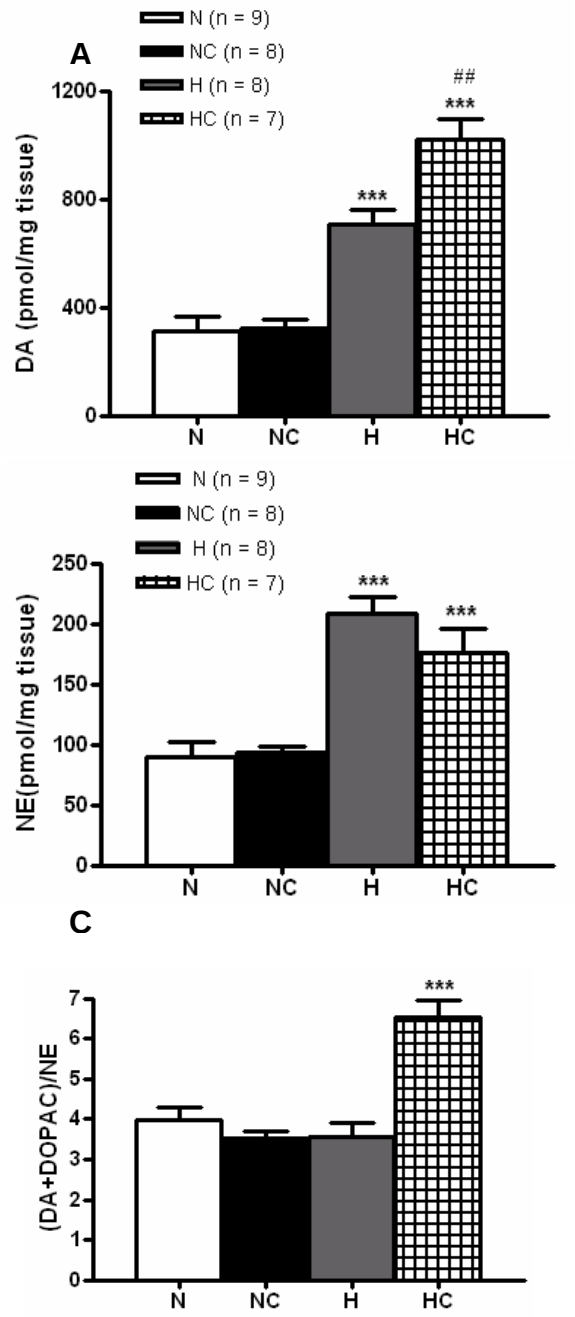


Figure 47 Effects of chronic caffeine intake, chronic hypoxia and both treatments applied together on the content of catecholamines and dopamine (DA)/norepinephrine (NE) ratios. **A** and **B** show absolute levels of DA and NE in the CBs of the distinct groups (N - normoxic rats, NC - normoxic rats plus chronic caffeine, H - chronic hypoxic rats, HC- chronic hypoxic rats with chronic caffeine ingestion. **C** shows that the DA/NE ratios increased only when the 2 chronic treatments were applied conjunctly. The dihydroxyphenyl acetic acid (DOPAC) content of the CB was included to estimate the ratios as it is the only CA catabolite present in significant amounts. Data represent means ± SEM of n individual values given in the drawing. One - Way ANOVA with Bonferroni multi-comparison test; *p<0.05, **p<0.01, ***p<0.001 corresponds to comparison made between the normoxic group and all the other groups. ## P<0.01 comparisons made between group H and group HC

Exposure of rats to chronic hypoxia for 15 days increased the DA content of CB by 126.8%, reaching a value of 705.7± 54.42 pmol/mg tissue (n =8). When rats were treated with both paradigms (group HC) the DA content increased respectively 228.2% and 44.7% when compared with normoxic rats and with chronic hypoxic rats (Figure 47A).

In all chromatograms a small amount of dihydroxyphenyl acetic acid (DOPAC, the main catabolite of DA), representing between 0.5 and 2% of the DA, was also detected (not shown) and was added to DA to calculate the ratio

(DA+ DOPAC/NE) (Figure 47C). NE levels were similar in normoxic rats and in the group NC with contents of 88.99 ± 13.03 pmol/mg tissue and 92.84 ± 5.9 pmol/mg tissue, respectively (Figure 47B). Chronic hypoxia also increased the levels of NE to 207.8 ± 14.58 pmol/mg tissue. However, contrary to DA, the exposure to both paradigms (group HC) did not cause significant statistical changes in NE levels when compared with group H. Figure 47C shows mean (DA+DOPAC)/NE ratios in all paradigms. It is evident that the rat CB is a dopaminergic organ in all paradigms studied, and that the dopaminergic quality increases when rats are exposed to chronic hypoxia conjunctly with chronic caffeine intake. Thus, in the CBs of the control animals the (DA+DOPAC)/NE ratio was 3.96 ± 0.32, this ratio being significantly increased and reaching values of 6.53 ± 0.42 in the organs of rats exposed to chronic hypoxia and chronic caffeine.

7.3.3. Effect of chronic caffeine intake on CA synthesis and CA turnover time in the carotid body of control and chronic hypoxic rats

The rate of ^{3}H-CA synthesis in CB of rats exposed to the different paradigms as well as the amount of free ^{3}H-tyrosine present in the tissue and the amount of ^{3}H-tyrosine incorporated into proteins, and the turnover time of DA and NE are shown in Figure 48. The rate of synthesis was calculated as described in Section 7.2.3. and the turnover time was estimated by dividing endogenous content by the rate of CA synthesis (i.e. pmol/CB)/[(pmol/CB)/hour] or pmol/mg tissue/[(pmol/mg tissue)/hour]. The rate of ^{3}H-NE synthesis in control CB was 1.82 ± 0.32 (n = 8) pmol/mg tissue/hour. In groups NC and H, a small, non-significant tendency to decrease to values of 1.27 ± 0.17 (n = 7) and 1.22 ± 0.19 (n = 6), respectively can be observed. In the group HC, the value shows a clear tendency to increase, reaching rates of 2.65± 0.36 (n = 8) pmol/mg tissue/hour in the CB, that was not significantly different from the control group (N), but was statistically different from group H.

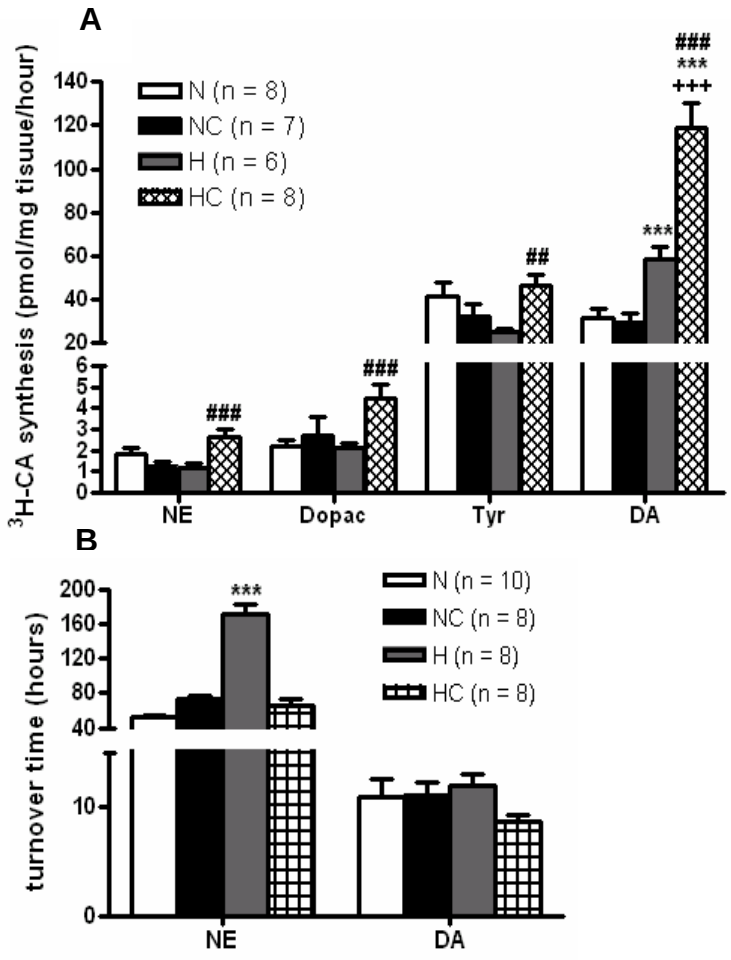


Figure 48 Effect of chronic caffeine intake, chronic hypoxia and both on **(A)** the synthesis rate of catecholamine (CA), and accumulation of the natural precursor tyrosine and **(B)** turnover time in the CB in normoxic animals (N) and in rats submitted to different paradigms (normoxic rats with chronic caffeine intake – NC; chronic hypoxic rats – H; chronic hypoxic rats with chronic caffeine intake – HC). ***P<0.01 Normoxic rats vs. all other paradigms; ##P<0.01,###P<0.001 H group vs. HC. Two-Way ANOVA with Bonferroni multi-comparison test. Data represent means ± SEM of *n* individual values given in the drawing.

A similar pattern was noticeable with the rate of appearance of ^{3}H-DOPAC and with the incorporation of free ^{3}H-tyrosine into the tissue. The pattern for the rate of synthesis of ^{3}H-DA was different since chronic hypoxia induced a statistically significant increase of 85.8%, reaching a value of 58.81 ± 5.06 pmol/mg tissue/hour (n = 6). The application of chronic caffeine conjunctly with chronic hypoxia produced a further increase in the rate of synthesis of ^{3}H-DA to a value of 118.58 ± 11.8 pmol/mg tissue/hour relative to control value and to the value of group H. In Figure 48B we present the turnover time for DA and NE with all paradigms. While, in the case of DA, no significant changes in the turnover time were observed, being around 9-12 h in all conditions tested, in the case of NE there was a statistically significant increase in the NE turnover time with chronic hypoxia exposure: in control CBs (group N) the turnover time was 52 h and in group H it reached 171 hours ($p < 0.001$). The application of the

both treatments together, chronic hypoxia and chronic hypoxia plus chronic caffeine return the NE turnover time to a value similar to the control (66 hours).

7.3.4. Effect of chronic caffeine intake on CA content, CA synthesis and CA turnover time in superior cervical ganglia of control and chronic hypoxic rats

To investigate whether these alterations observed in the CA metabolism: content, synthesis and turnover time, and in the (DA+DOPAC)/NE ratio were specific to the CB, we performed the same experiments in SCGs in the same experimental conditions.

Figure 49 shows mean contents of CA, DA and NE in SCGs and the ratio (DA+DOPAC)/NE in control rats (group N) and in rats exposed to chronic hypoxia, chronic caffeine or both. Figure 49A and B shows that exposure to chronic hypoxia did not modify the levels of DA and NE in the SCG. In the same manner, the exposure to both paradigms conjunctly (group HC) did not modify the levels of both CA when compared with groups N and H.

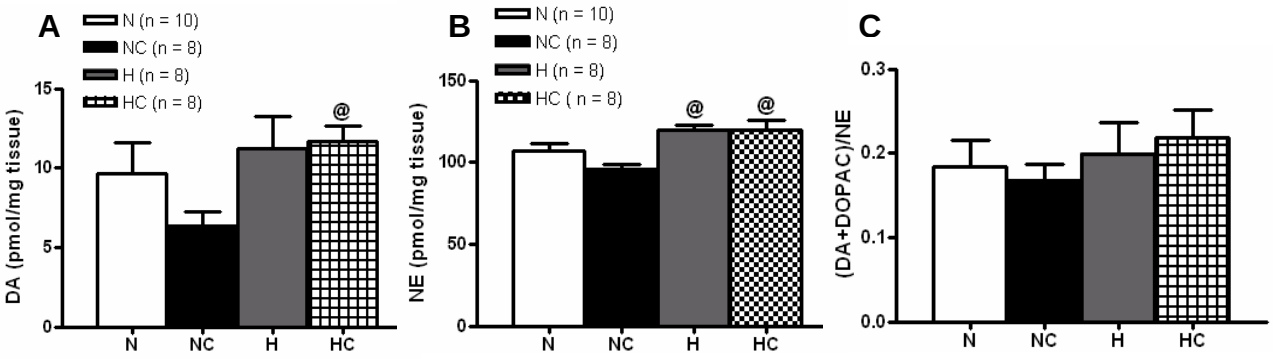


Figure 49 Effects of chronic caffeine intake (NC), chronic hypoxia (H) and both treatments (HC) on the content of catecholamines (CA) and on dopamine (DA)/norepinephrine (NE) ratios in SCGs. **A** and **B** represent absolute levels of DA and NE in the SCGs. N - normoxic rats. **C** DA/NE ratios for the different paradigms. The dihydroxyphenyl acetic acid (DOPAC) content of the SCG was included to estimate the ratios as it is the only CA catabolite present in significant amounts. Data represent means ± SEM of n individual values given in the drawing. @P<0.05, corresponds to comparison between the NC group and all the other groups; One-way ANOVA with Bonferroni multi-comparison test.

The exposure to a chronic caffeine treatment in normoxic rats produced a decrease in both DA and NE levels in SCGs that was not statistically different from the controls, but was significantly different from the values in groups H and HC. Figure 49C shows the (DA+DOPAC)/NE ratios in SCGs in response to all paradigms. It can be observed that SCG is preferentially a noradrenergic organ, and that the exposure to chronic hypoxia, chronic caffeine or both treatments applied together does not modify the (DA+DOPAC)/NE ratios.

The ^{3}H-CA rate of synthesis and the turnover time for NE and DA in SCGs in all paradigms is represented in Figure 50. The chronic intake of caffeine did not modify the rate of synthesis of either NE or DA (Figure 50A), but produced an increase in the turnover time of NE and decreased the DA turnover time (Figure 50B).

Chronic hypoxia exposure as well as the intake of caffeine conjunctly with chronic hypoxia did not modify either the rate of synthesis of DA or NE, or the turnover time of DA in SCGs. However, both paradigms increased the turnover time of NE (Figure 50A and B). The absence of significant alterations in the DA metabolism in SCGs in all the experimental conditions tested would indicate that the alterations in DA metabolism produced by chronic hypoxia and by the ingestion of caffeine during chronic hypoxia are characteristic of CB.

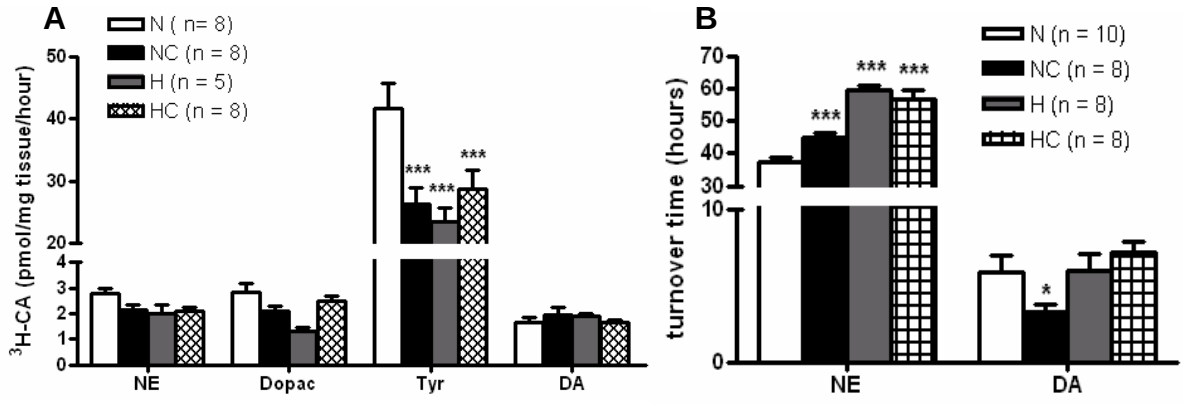


Figure 50 Effects of chronic caffeine intake (NC), chronic hypoxia (H) and both treatments (HC) on **(A)** the synthesis rate of catecholamines (CA), and accumulation of the natural precursor tyrosine and **(B)** turnover time in the SCGs in normoxic animals and in rats submitted to the different paradigms. *P<0.05, ***P<0.01 Normoxic rats vs. all other paradigms; ##P<0.01, ###P<0.001 H group vs. HC. Two-Way ANOVA with Bonferroni multi-comparison test. Data represent means ± SEM of *n* individual values given in the drawing.

7.3.5. Effect of chronic caffeine intake on DA, ATP and adenosine release evoked by acute hypoxia, from carotid body in control and chronic hypoxic rats

Figure 51 illustrates the effects of chronic caffeine intake, chronic hypoxia and both treatments applied together on the basal release of endogenous DA, ATP and adenosine from rat CB. In the protocol used to study the effect of acute hypoxia on the release of these neurotransmitters, in addition to the periods correspondent to hypoxic stimulus application, we incubated the CBs for 4 periods of 10 min in 20% O_2 or 12% O_2 respectively, depending on whether the rats were normoxic or hypoxic (control values), and decided to selected the second period of 20% O_2 or 12% O_2 (C1, period 2 of the protocol described in Section 7.2.3) to calculate the basal release of these neurotransmitters, since the first one is considered a recovery period that allows CBs to recover from the surgery.

Figure 51A shows that chronic hypoxia increased the basal release of DA from rat CB by 145.9% relative to the control value (49.47 ± 6.52 pmol/mg tissue, n = 6). Chronic caffeine intake did not modify the basal release of DA from the CB either in normoxic rats or in chronic hypoxic rats.

A different pattern was noticeable for the effect of chronic caffeine intake on the release of ATP from the CB of normoxic and chronic hypoxic rats (Figure 51B). Basal value of ATP released from CB in normoxic rats was 81.55 ± 13.71 pmol/mg tissue (n = 6). A tendency to increase to a value of 108.40 ± 8.01 pmol/mg tissue (n = 8) was observed for ATP basal release in normoxic rats submitted to chronic caffeine ingestion. Nevertheless this increase was not significant. Chronic hypoxia did not modify the basal release of ATP from rat CB, and this treatment applied in conjunction with chronic caffeine intake was also incapable of modifying ATP basal release (Figure 51B).

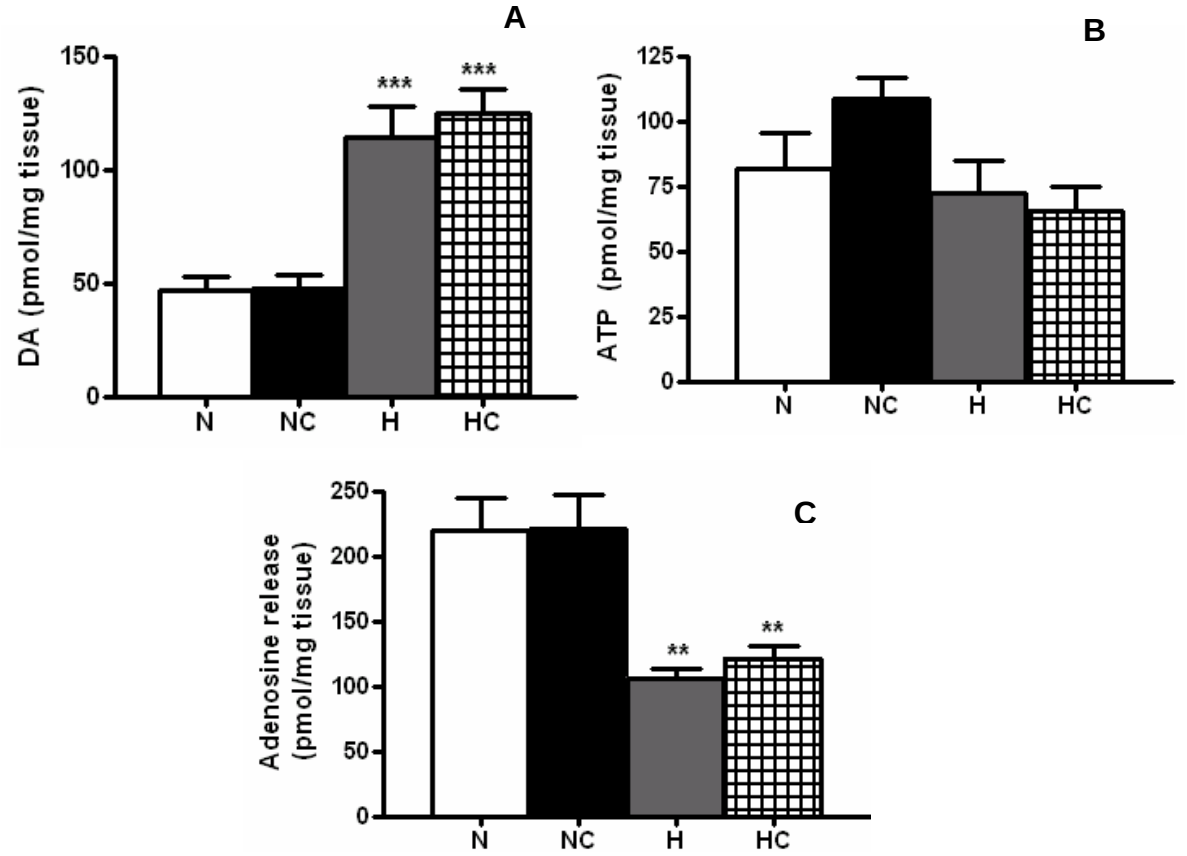


Figure 51 Effects of chronic caffeine intake (NC), chronic hypoxia (H) and effect of both treatments applied together (HC) on the basal endogenous release of DA **(A)**, ATP **(B)** and adenosine **(C)** from rat CBs in comparison with normoxic (N) animals (n = 6). NC (n = 7); H (n = 5); HC (n = 6). Note that basal release corresponds in normoxic rats to the release in response to 20% O_2 and in chronic hypoxic animals to the release in response to 12% O_2. *P<0.05, ***P<0.01 Normoxic rats vs. all other paradigms. One-Way ANOVA with Bonferroni multi-comparison test. Data represent means ± SEM.

The pattern of basal adenosine release contrasts with the release of DA and ATP (Figure 51C). Chronic caffeine intake in normoxic rats did not modify the basal release of adenosine when compared with control rats (220.6 ± 26.15 pmol/mg tissue, n = 6 vs. 220.0 ± 224.05 pmol/mg tissue, n = 7). However, chronic hypoxia exposure decreased the release of adenosine from the CB by 51.7 %. The chronic caffeine intake applied conjunctly with chronic hypoxia did not modify the basal levels of adenosine present in extracellular medium relative to the levels found in group H.

Figures 52, 53 and 54 show the effects of acute hypoxia on the release of DA, ATP and adenosine in control CB and in CB submitted to the different paradigms. Note that the basal release of DA diminished from C1 to C2 (see experimental protocol in Section 7.2.4., i.e. after the 1st acute hypoxic stimulus

in all the experimental conditions studied. In contrast, the levels of adenosine and ATP released in basal conditions were similar in C1 and C2.

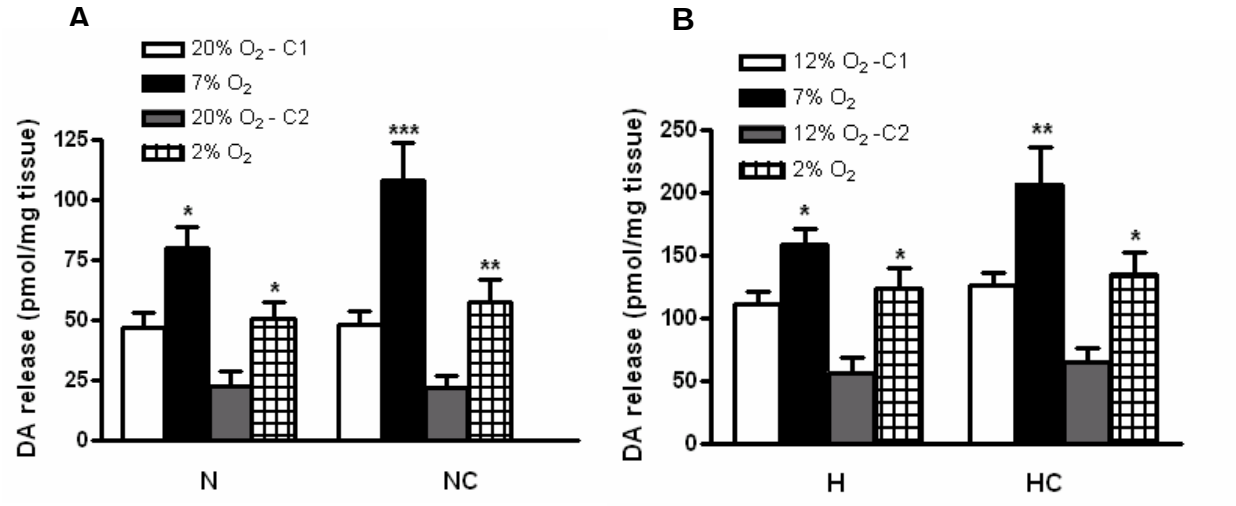


Figure 52 Effects of chronic caffeine intake (NC and HC) on the endogenous release of dopamine (DA) from CB in response to acute hypoxia (7% and 2% O_2) in rats submitted to (**A**) normoxia (N and NC) and (**B**) chronic hypoxia (H and HC). C1 and C2 represent basal values before the application of the acute hypoxic stimulus, corresponding to the release of DA from CB in response to 20% O_2 in normoxic rats and 12% O_2 in chronic hypoxic rats. N (n = 6); NC (n = 7); H (n = 5); HC (n = 6). * P<0.05, **P<0.01; Two-Way ANOVA with Bonferroni multi-comparison test corresponding to the comparison with C1 or C2.

Concerning the release of endogenous DA in response to acute hypoxia, it can be observed that 7% O_2 produces a statistically significant increase of 72.1% and 43.4 % in the release of DA from CB of both control rats (80.00 ± 19.1 pmol/mg tissue) and rats exposed to chronic hypoxia (157.8 ± 30.41 pmol/mg tissue), respectively. However, chronic caffeine intake produced higher statistically significant increases of 125.8% and 64.6% in the DA released from CB in normoxic and chronically hypoxic rats in response to 7% O_2 (Figure 52A and B). In response to a more intense hypoxic stimulus, 2% O_2, chronic hypoxic exposure does not modify the increase in the release of DA from CB when compared with control values (123.36% increase in controls vs. 119.17% increase in chronic hypoxic rats).

The treatment with chronic caffeine did not alter the release of DA evoked by 2% O_2 in control rats and in rats exposed to chronic hypoxia (Figure 52B). These results are in agreement with the acute effects of caffeine on

peripheral chemoreceptors described in Chapter 3 and suggest that caffeine and thereby adenosine receptors are involved in the response to moderate hypoxias, that is, hypoxias that are compatible with life.

The effects of chronic caffeine intake and chronic hypoxia on the release of ATP evoked by acute hypoxia (7% and 2% O_2) are represented in Figure 53A and B. It can be observed that moderate (7% O_2) and intense hypoxia (2% O_2) increase the release of ATP from the CB of control rats by 73.0% and 88.1%, respectively. Chronic caffeine ingestion did not modify the evoked release of ATP from rat CB in any of the hypoxias studied, increasing the release in response to 7% O_2 by 68.9% and in response to 2% O_2 by 77.5 % (Figure 53A).

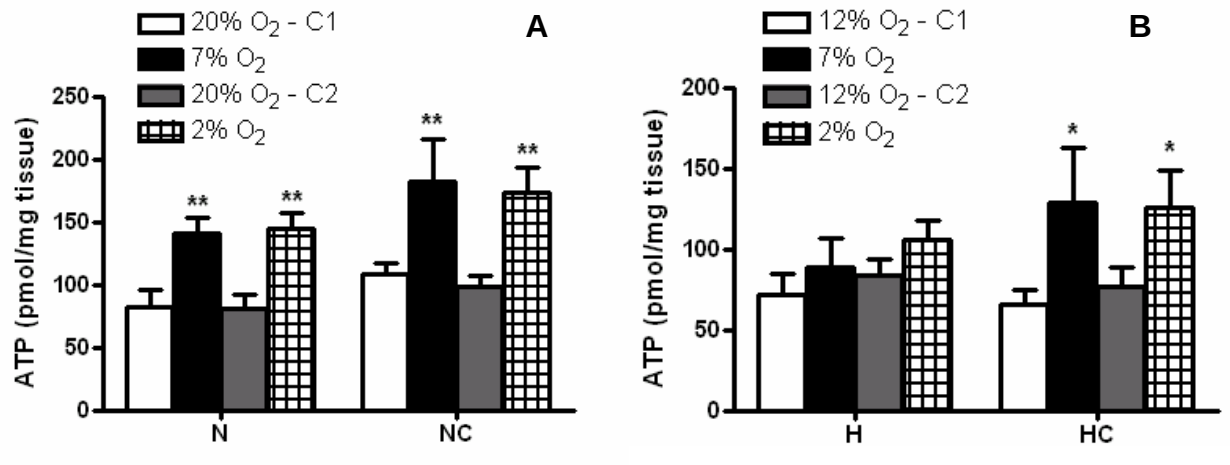


Figure 53 Effects of chronic caffeine intake (NC and HC) on the release of ATP from CB in response to acute hypoxia (7% and 2% O_2) in rats submitted to (**A**) normoxia (N and NC) and to (**B**) chronic hypoxia (H and HC). C1 and C2 represent basal values before the application of the acute hypoxic stimulus, corresponding to the release of ATP from CB in response to 20% O_2 in normoxic rats and 12% O_2 in chronically hypoxic rats. N (n = 6); NC (n = 7); H (n = 5); HC (n = 6). * P<0.05, **P<0.01; Two-Way ANOVA with Bonferroni multi-comparison test corresponding to the comparison C1 or C2. Data represent means ± SEM.

The pattern of ATP release induced by acute hypoxia was very distinct in CBs from rats submitted to 15 days of chronic hypoxic exposure. As can be observed in Figure 53B, neither 7% O_2 nor 2% O_2 were sufficient stimuli to evoke a significant release of ATP from CB in chronic hypoxic rats, suggesting that ATP is not a crucial neurotransmitter for the response to acute hypoxia during chronic hypoxic exposure. Chronic caffeine ingestion during chronic hypoxia exposure revert the effect of chronic hypoxia on the release of ATP

evoked by acute hypoxic stimulus (Figure 53B). Acute 7% O_2 and 2% O_2 induced an increase of 96.2% and 63.6% in ATP release from CB of rats treated with chronic caffeine during hypoxic exposure.

Figure 54 are illustrates the effects of chronic hypoxia and chronic caffeine intake and both paradigms applied together on the release of adenosine from CB evoked by 2 acute hypoxias of distinct intensity, 7% and 2% O_2. It can be observed, as previously described by Conde and Monteiro (2006) in controls rats, that moderate hypoxias release more adenosine than high intensity hypoxias, the values not having any statistical difference (Figure 54A). Nevertheless, it can be observed that in chronic hypoxic conditions the increase in adenosine release from CB is proportional to the intensity of acute hypoxia applied (Figure 54B).

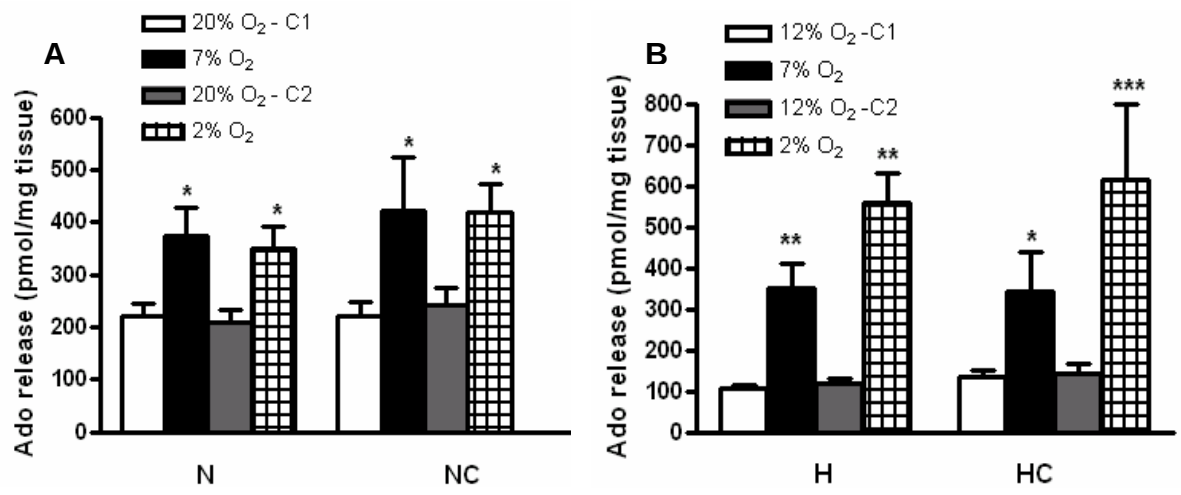


Figure 54 Effects of chronic caffeine intake (NC and HC) on the release of adenosine from CB in response to acute hypoxia (7% and 2% O_2) in rats submitted to (**A**) normoxia (N and NC) and to (**B**) chronic hypoxia (H and HC). C1 and C2 represent basal values before the application of the acute hypoxic stimulus, corresponding to the release of adenosine from CB in response to 20% O_2 in normoxic rats and 12% O_2 in chronic hypoxic rats. N (n = 6); NC (n = 7); H (n = 5); HC (n = 6) * P<0.05, **P<0.01; Two-Way ANOVA with Bonferroni multi-comparison test corresponding to the comparison with C1 or C2. Data represent means ± SEM.

As can be observed in Figure 54B, in chronic hypoxia, CB releases more adenosine, in response to an acute hypoxic stimulus, than in control conditions: increases of 230.95% and 372.0% in the release of adenosine, in response to 7% and 2% O_2, respectively, were observed. However, these increases can be

due to the reduction of adenosine basal levels. Chronic caffeine intake produced non-significant higher increases of 100.9% and 73% in the release of adenosine evoked by 7% and 2% O_2 from the CBs of normoxic rats. However, the ingestion of caffeine applied conjunctly with chronic hypoxic exposure produced non-significant smaller increases in the release of adenosine by the acute hypoxia (153.74% in response to 7% O_2 and 335.88% in response to 2% O_2) when compared with chronic hypoxic rats.

7.3.6. Effect of chronic caffeine intake on tyrosine hydroxylase expression in the carotid body of control and chronic hypoxic rats

The mRNA coding for TH is upregulated in CB chemoreceptor cells within the first few hour of hypoxia (Czyzk-Krzeska et al., 1992) increasing approximately 3 times in the CB with chronic hypoxia exposure (Wang et al., 1998; Bisgard, 2002; Ganfornina et al., 2005). Kobayashi and Milhorn (1999) postulated that adenosine could modulate CB gene expression and since caffeine is an antagonist of adenosine receptors it would be expected that chronic caffeine intake as well as the application of chronic hypoxia with chronic caffeine in the rats would alter the expression of TH in the CB.

We decided to use a semi-quantitative technique, Western-Blot, to study the effect of chronic caffeine intake on the expression of TH in the CB of normoxic and chronic hypoxic rats.

As illustrated in Figure 55, using mouse anti-TH antibody, and expressing the TH immunoreactivity relative to the immunoreactivity to ß-actin, we found that chronic caffeine intake induced a non-significant statistical increase in TH expression in normoxic CBs ($P = 0.1$). It can be observed that chronic hypoxia increased the TH expression in the CB 2.8 times, which is in agreement with the data previously found by Wang et al. (1998). Ingestion of caffeine during the chronic hypoxia exposure produced a higher increase (3.3 times) relative to the controls, but not significantly different from the value obtained in chronic hypoxia. These results demonstrate that chronic caffeine did not induce significant alterations in the expression of TH in either normoxic or chronic hypoxic rats.

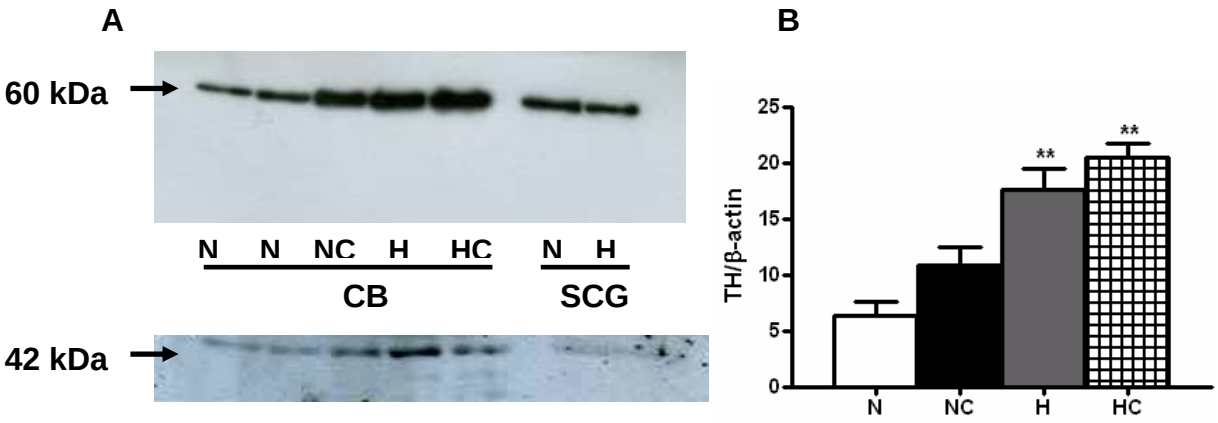


Figure 55 Tyrosine hydroxylase (TH) immunoreactivity in the CB and in SCGs in control rats (N) and in rats submitted to different treatments: chronic caffeine intake (NC), chronic hypoxia exposure (H) and chronic caffeine + chronic hypoxia (HC). **A** Western Blot comparing TH immunoreactivity, corresponding to 60 KDa band, when comparing CBs and SCGs (controls and submitted to different paradigms). A re-probing of the membranes with an anti-ß-actin antibody, corresponding to the 42 KDa band is shown below the gel for TH. **B** shows the average relative TH immunoreactivity in the CB (n= 3) in the different paradigms expressed in relation to ß-actin immunoreactivity. ** P<0.01; One-Way ANOVA with Dunnett's multi-comparison test, comparing all the groups with control group. Data represent means ± SEM.

7.3.7. Effects of chronic caffeine intake on carotid sinus nerve activity evoked by hypoxia and hypercapnia in control and chronic hypoxic rats

The next group of experiments was designed to study the effect of chronic caffeine intake, chronic hypoxic exposure and both paradigms applied together on the overall output of the CB measured as CSN chemosensory activity. Figure 56 illustrates the results obtained in all the experimental conditions cited above. Although it is not evident from Figure 56 A and B that chronic hypoxia increased the basal CSN chemosensory activity, mean basal CSN chemosensory activity increased 80.64% with chronic hypoxia exposure (Figure 56C). Chronic caffeine intake did not modify the basal CSN chemosensory activity in either normoxic or chronic hypoxic animals (Figure 56C). Mean basal activity for each paradigm was: control - 5.42 ± 0.81 impulses/s; normoxic rats with chronic caffeine ingestion – 5.10 ± 0.66 impulses/s; chronic hypoxic rats – 9.76 ± 0.89 impulses/s; chronic hypoxic rats with chronic caffeine ingestion – 8.76 ± 0.61 impulses/s.

In order to correct the activity of CSN in response to any given stimulus, the activity was expressed as times over basal.

At the onset of this section we would like to mention that the values of % of O_2 in the recordings of the CSN activity do not correspond to the values of O_2 for the *in vitro* experiments. This difference is based on the exchange of gases that exists between the superfusion medium and the atmosphere in the opened chamber where the CSN activity is recorded. For example, 2% O_2 corresponds to a PO_2 of ≈ 22 mmHg, but Tyrode solution equilibrated with 0% O_2 in the superfusion chamber is at a PO_2 of ≈ 25 mmHg (Conde et al., 2006a).

Figure 56D shows that exposure to chronic hypoxia did not modify the activity of CSN in response to moderate and intense hypoxia, 5% and 0% O_2, respectively, but the response to hypercapnic-acid stimulus (20% CO_2, pH = 6.8) was significantly reduced by 49%. It can be observed that chronic caffeine intake in normoxia produced a tendency to augment the responses to hypoxia, inducing non-significant increases of 25.5% and 33.6% in the activity of CSN in response to moderate and intense hypoxia, 5% and 0% O_2, respectively.

However, treatment with chronic caffeine does not modify the CSN chemosensory activity induced by hypercapnia. Chronic caffeine applied conjunctly with chronic hypoxia clearly attenuates the CSN chemosensory responses both to hypoxia and to hypercapnia.

As can be observed in Figure 56D the application of both paradigms significantly reduces the response to 5% O_2, by 61.2% and 55.0% in relation to normoxic and chronic hypoxic rats, respectively. CSN chemosensory activity in response to 0% O_2 was significantly inhibited by 66.8% and 61.9% when compared with normoxic and chronic hypoxic rats. The response to hypercapnia was almost completely abolished, the values being reduced by 87.2% and 75.2% in comparison to normoxic and chronic hypoxic rats. These results indicate that chronic application of caffeine also has an inhibitory effect on CSN activity that is even higher in chronic hypoxic rats than when applied acutely in normoxic rats.

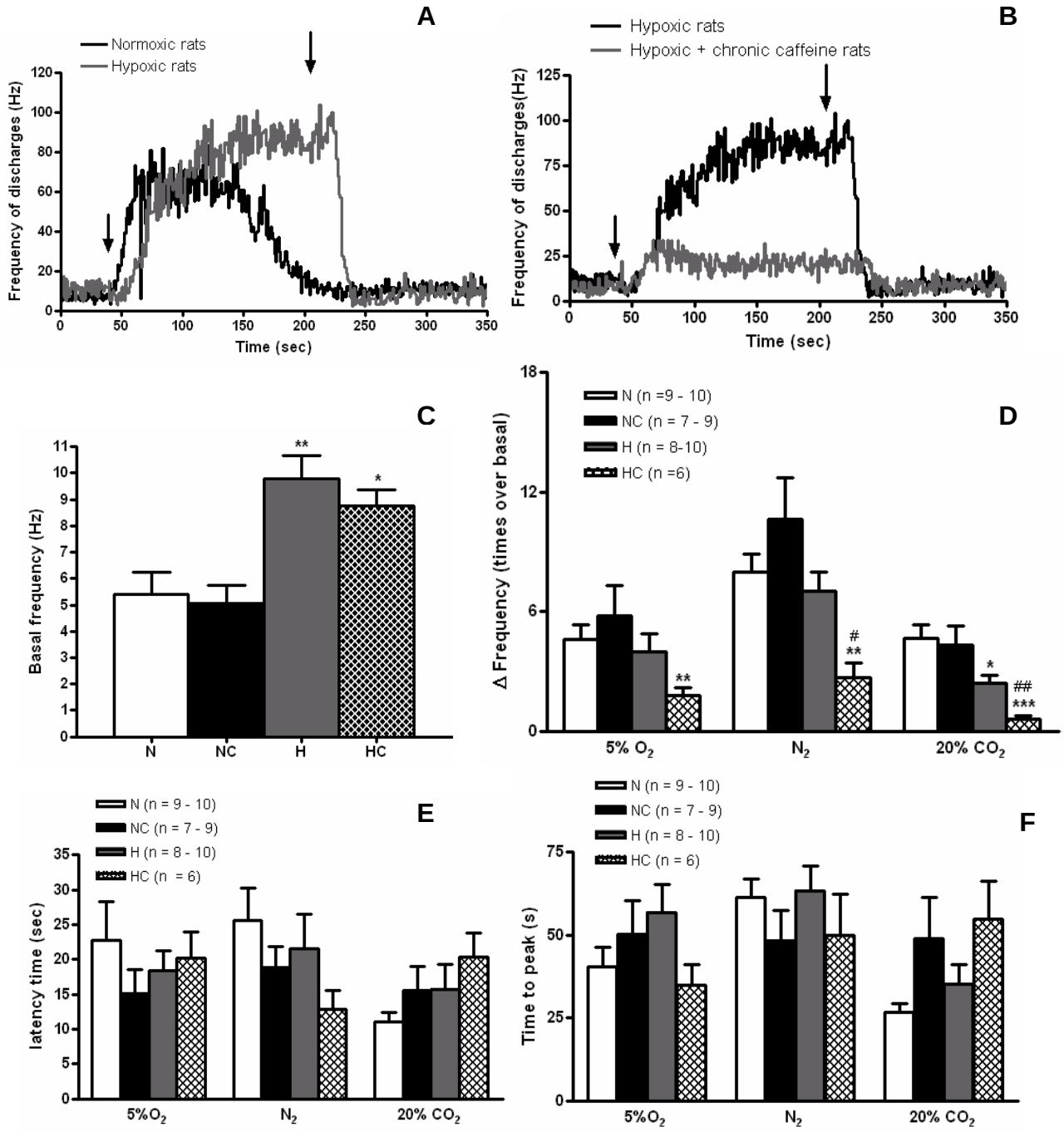


Figure 56 Effects of chronic caffeine intake on the carotid sinus nerve (CSN) activity in normoxic and chronic hypoxic rats. **A**) Typical recording of the frequency of action potentials of CSN in response to N_2 in normoxic and chronic hypoxic rats. **B)** Typical recording for the effect of chronic caffeine ingestion on the frequency of actions potentials of CSN in response to N_2 in chronic hypoxic rats. Arrows indicate the period of application of acute hypoxic stimulus (N2). Panels **C**, **D**, **E** and **F** represent, respectively, means of basal CSN activity, means of increases in peak frequencies, latency times and time to peak in control rats (N), in normoxic rats with chronic caffeine ingestion (NC), in rats exposed to chronic hypoxia (H) and in chronic hypoxic rats after chronic caffeine ingestion (HC). Data represent means ± SEM of n individual values given in the drawing. *P<0.05, **P<0.01, ***P<0.001 vs. control values; #P<0.05, ##P<0.01 vs. chronic hypoxic values

For each given stimulus we also measured the latency of the response and the time to peak response. The latencies range from 15 to 22 sec for moderate hypoxia, 13 to 25 sec for intense hypoxia and from 12 to 20 for hypercapnia (Figure 56E). The times to peak ranged between 35 and 56 sec in moderate hypoxia, 48 and 63 sec in intense hypoxia and 27 and 54 sec in hypercapnia (Figure 56F). No statistical differences in these two parameters were found between all the paradigms studied.

7.3.8. Effects of chronic caffeine intake on the ventilatory responses induced by hypoxia and hypercapnia in control and chronic hypoxic rats

The next group of experiments was designed to assess the effect of chronic caffeine intake on the ventilatory responses (frequency, tidal volume and minute volume) to hypoxia and hypercapnia in control and chronic hypoxic rats.

Since some controversies exist about the sensitisation phenomena and the period of time to its appearance (see Introduction) we decided to study the ventilatory responses to hypoxia and hypercapnia in rats submitted to 8 and 15 days of chronic hypoxia.

In figure 57A we can observe that in control animals the breathing frequency increased with increasing intensity of hypoxia (12% and 10% O_2), except for the highest hypoxic intensity (7% O_2) tested, where a small reduction in the response can be seen. The same pattern was observed with exposures of 8 and 15 days to chronic hypoxia although breathing frequencies in 12% O_2 decreased with the increasing time of hypoxic exposure. At 8 days of hypoxic exposure the breathing frequency decreased significantly by 19% in 12% O_2 compared with the control response for the same hypoxic intensity (128.17 ± 3.75 bpm); at 15 days of hypoxia the response diminished significantly by 33% to a value of 86.22 ± 1.78 bpm, these values being very similar to those obtained in the control rats in 20% O_2 (84.91 ± 1.73 bpm). The breathing frequencies in response to acute hypoxia of 10% and 7% O_2 in rats exposed to 8 days of hypoxia were not significantly modified when compared with the same responses in control rats. However, a clear tendency towards an increase in the breathing frequency in response to 7% O_2 was observed (Figure 57A),

suggesting an increased sensitivity to high intensity acute hypoxias in the rats exposed to 8 days of chronic hypoxia. As can be seen in Figure 57B, the tidal volume in the several O_2 intensities tested does not change significantly in any of the paradigms i.e. after 8 or 15 days of hypoxic exposure, although, with chronic hypoxia, a tendency towards an increase in response to acute hypoxic challenges, mainly after 15 days, can be observed. When the ventilatory responses are analysed in terms of minute volume (VE) (Figure 57C) and corrected to the rat's weight, the non-significant increases in acute hypoxia observed in tidal volume became significant.

Basal values for chronic hypoxic animals (values during 12% O_2) are even higher (773.93 ± 56.61 (ml/min)/kg) than the value of VE for normoxic rats in response to the same hypoxic challenge (702.52 ± 29.09 (ml/min)/kg). It can also be noted that the pattern of increase in minute ventilation with the increasing hypoxic intensity observed in control rats was not modified in any of the chronic hypoxias tested (8 and 15 days), being even more accentuated in both chronic hypoxias. Therefore, we can say that chronic hypoxic rats showed an increase in ventilation in both basal conditions and in response to acute hypoxia. Concerning the response to acute hypercapnia, rats exposed to 8 days of hypoxia exhibited a statistically significant increase of 46.7% in minute ventilation when compared to control rats (1081 ± 62.35 (ml/min)/kg, n = 14). However, exposure to 15 days of hypoxia reverted this sensitisation to a value of 1253 ± 108.7 (ml/min)/kg (n = 10).

All these data taken together suggest that acclimatisation of the chemoreceptors to hypoxia was already apparent after 1 week of chronic hypoxia but was more accentuated after 2 weeks, as hypoxic ventilatory response is increased at this period of time (Figure 57C).

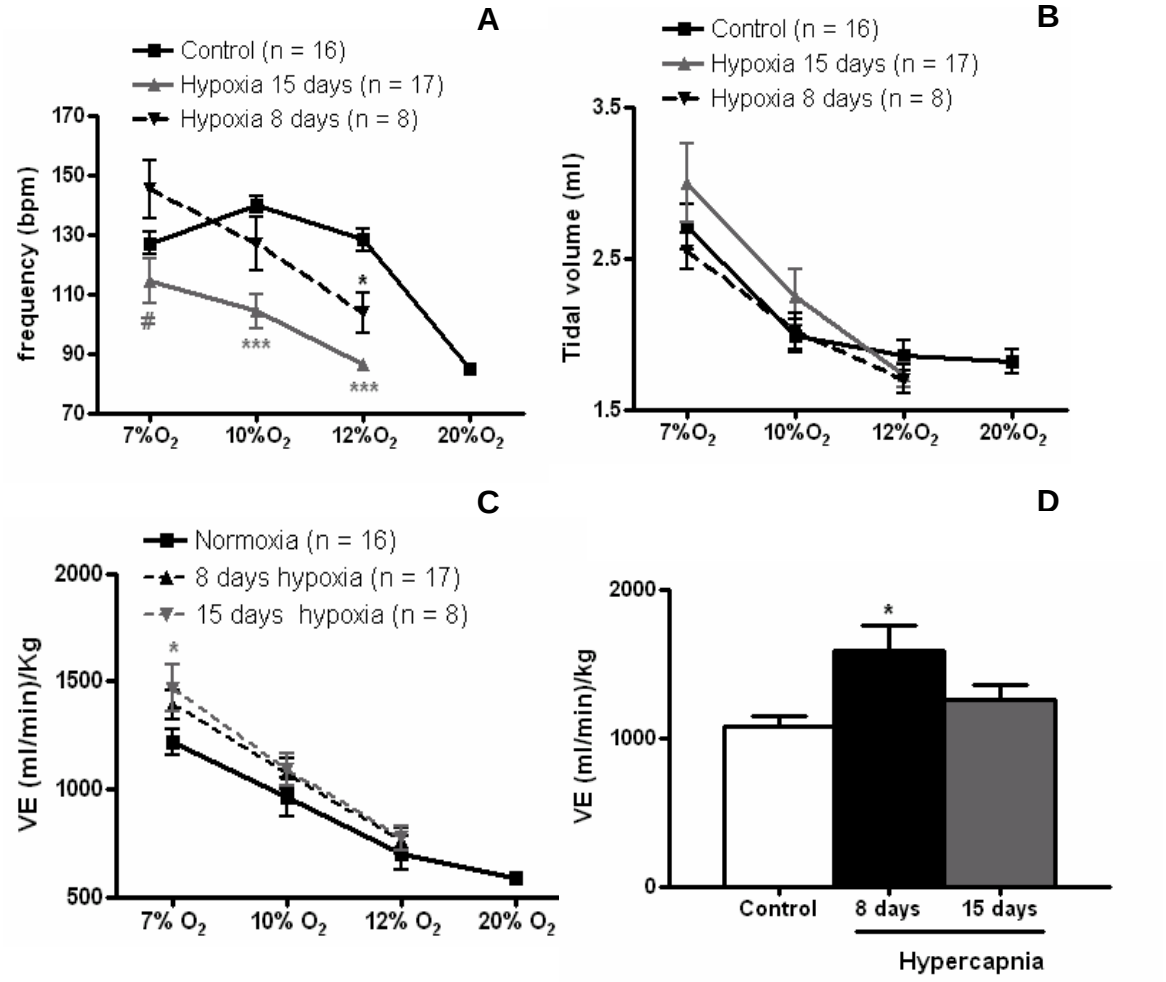


Figure 57 Effect of 8 and 15 days exposure to hypoxia (12% O_2) on the ventilatory responses to acute hypoxia (10 and 7% O_2) and hypercapnia (5% CO_2). Ventilatory responses to acute hypoxia were assessed as (**A**) frequency, (**B**) tidal volume and (**C**) minute volume (VE). **D**) Effect of acute application of hypercapnia on minute ventilation in control rats and in rats exposed to 8 and 15 days of hypoxia. Acute hypoxias and hypercapnia were applied for 10 minutes in accordance with the protocols described in Section 7.2.8. Minute volume values were corrected to the rat's weights. *P<0.05 vs control values; #P<0.05 8 days hypoxic exposure vs. 15 days of hypoxic exposure. Data represent means ± SEM.

The fact that caffeine is an active substance widely consumed across the world, and that at concentrations ingested by humans it antagonises adenosine receptors, led us to study the effect of chronic caffeine intake on ventilation in control and chronic hypoxic animals. The effect of chronic caffeine intake was studied in the ventilatory responses to several hypoxic intensities, 12%, 10% and 7% O_2 in normoxic rats and 10% and 7% O_2 in rats submitted to a atmosphere of 12% O_2 (12% O_2 in chronically hypoxic animals is considered the basal value) and in response to acute hypercapnia, 5% CO_2. In normoxic rats,

chronic caffeine intake during 8 days did not produce any alterations in the pattern of hyperventilation in response to acute hypoxias, when compared with control rats (Figure 58A). Chronic ingestion of caffeine for 15 days in normoxic rats seems to follow the same pattern except for a facilitation of the response to a high intensity hypoxia, 7% O_2, with the chronic caffeine intake producing a significant increase of 34.8% in minute ventilation, when compared with the same response to 7% O_2 in control rats (Figure 58A). This result means that chronic caffeine intake can facilitate the ventilatory responses to high intensity hypoxias in normoxic subjects. In animals that were exposed to an atmosphere of 12% O_2 for 8 or 15 days, the chronic intake of caffeinedid not modify the ventilatory responses to the different acute hypoxias tested in any of the groups (Figure 58B).

Figure 59 shows the effect of chronic caffeine intake (8 or 15 days) on the ventilatory responses to hypercapnia, 5% CO_2, in normoxic rats and in rats submitted to 8 and 15 days of chronic hypoxia.

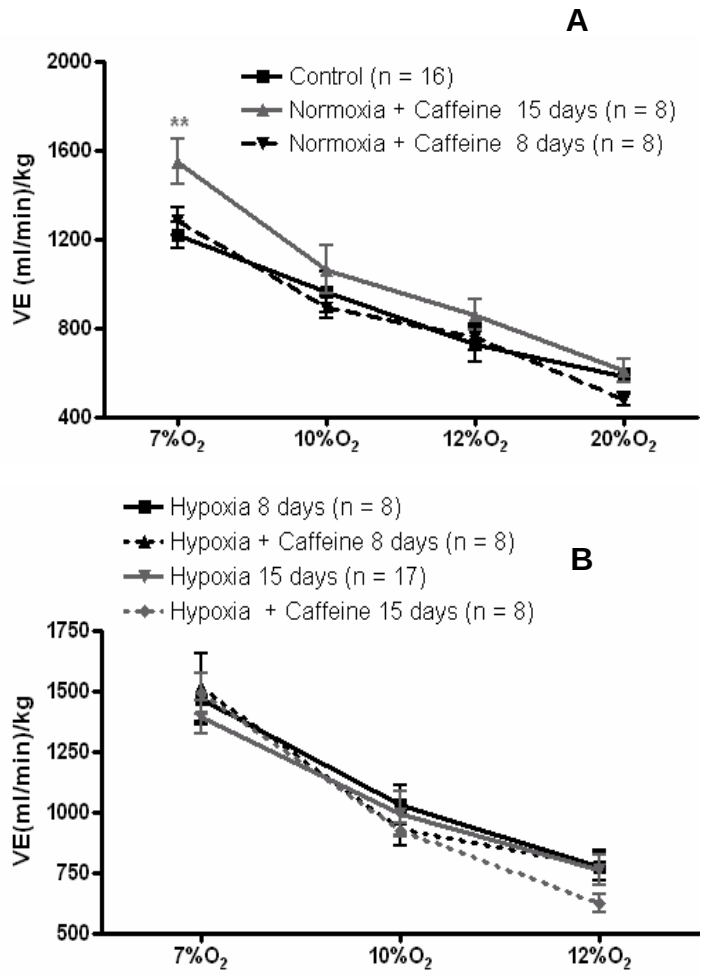


Figure 58 Effects of chronic caffeine intake on minute volume (VE) in response to acute hypoxias of several intensities (12%, 10% and 7% O_2) in normoxic animals (**A**) and in animals submitted to chronic hypoxia for 8 and 15 days (**B**). Acute hypoxias were applied for 10 minutes in accordance with the protocols described in Section 7.2.8. Basal value for hypoxic animals was considered to be the V_E in 12% O_2. **P<0.01; chronic caffeine intake for 15 days vs. controls. Data represent means ± SEM.

It is evident that chronic caffeine (8 or 15 days) intake did not significantly modify minute volume either in normoxic rats (Figure 59A) or in rats exposed to chronic hypoxia for 8 days. Nevertheless, the intake of caffeine in rats exposed to an atmosphere of 12% O_2 for 15 days significantly increased minute volume in response to acute hypercapnia by 57.9% (Figure 59B), suggesting that hypoxic ventilatory responses were not altered by chronic caffeine consumption in chronic hypoxic rats but hypercapnic ventilatory responses were.

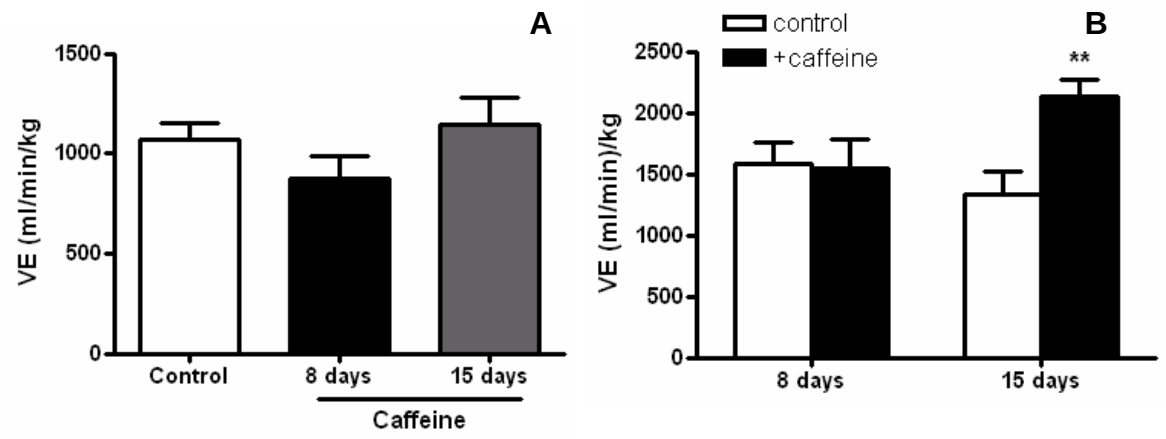


Figure 59 Effects of chronic caffeine intake on minute volume (VE) in response to hypercapnia (5%CO_2) in (**A**) normoxic rats and in (**B**) rats exposed to an atmosphere of 12% O_2 for 8 and 15 days. (n = 6-9) Hypercapnia was applied for 10 min in accordance with the protocol described in Section 7.2.8. **P<0.01; chronic caffeine intake for 15 days vs. control. Data represent means ± SEM.

7.4. Discussion

In this section we used a combination of neurochemical and physiological techniques to evaluate CB output measured as CSN chemosensory activity and ventilatory responses, as well as CB neurotransmitter (ATP, DA and adenosine) dynamics in chronic hypoxia. These methodologies allowed us to conclude that in chronic hypoxia: O_2 sensitivity is increased; that the exposure to this paradigm elicits acclimatisation; and that adenosine is essential for the CB chemoreception in chronic hypoxia, being one of the neurotransmitters involved in the increased O_2 sensitivity and contributing to the VAH.

In all the experimental protocols, chronic hypoxia was applied for a period of 15 days, although increases in ventilatory hypoxic response were

observed after an 8-day exposure to chronic hypoxia. However, our paradigm of 15 days of chronic hypoxia was validated i.e. after this period of exposure we achieved an increased CB O_2 sensitivity, as ventilatory hypoxic response, as well as CSN basal discharges in response to hypoxia, was increased.

The effects of chronic caffeine intake were also studied as a tool to evaluate the contribution of adenosine receptors/adenosine to the increased O_2 sensitivity observed in chronic hypoxia. At the outset of the discussion we should state that we have attributed all the effects of chronic caffeine ingestion during chronic hypoxia to an inhibition of adenosine receptors, since the concentrations of caffeine ingested are compatible with this inhibition and not with other cell targets (Section 1.7., Figure 14). In fact, for the inhibition of the other cellular caffeine targets, such as phosphodiesterases and ryanodine receptors, toxic doses of caffeine that are not compatible with life are needed. In accordance with our statements, all the effects of caffeine observed imply that, in all cases, they are due to an inhibition of adenosine receptors and therefore, in the rest of the discussion we will not make a specific distinction between the effects of caffeine or adenosine.

Since nothing is known about chronic effects of caffeine on the activity of CB chemoreceptors, in this chapter we have used chronic caffeine consumption in order to permanently block adenosine receptors in the CB and to obtain a situation that is similar to what happens with the long-term consumption of this xanthine in humans, predominantly in the occidental countries (see Introduction, Section 1.7).

At the onset of the discussion we would also like to mention that with the different experimental approaches used to measure the CB output, CSN chemosensory activity and ventilation, we can assess the contribution of the central nervous system to the responses in chronic hypoxia. We also studied the effects of chronic hypoxia and chronic caffeine on neurotransmitter dynamics. Since DA is one of the CB's better-characterised neurotransmitters, a detailed characterisation of CA dynamics (endogenous release, content, synthesis, turnover time) in chronic hypoxia and after/with chronic caffeine consumption was performed in the current study. In an attempt to clarify the importance of ATP in chronic hypoxia as suggested by He et al. (2006) and to

investigate the role of adenosine in this situation, the endogenous release of both neurotransmitters was studied.

In an attempt of simplify the discussion of this chapter the results are going to be summarised in a table and discussed as followed: 1) effects of chronic hypoxia 2) and the effects of chronic caffeine ingestion in control and chronic hypoxic animals.

7.4.1. Effects of chronic hypoxia on CB function

This study showed that chronic exposure to hypoxia elicits acclimatisation, as hypoxic ventilatory response (HVR) is increased and the CSN discharges, both basal and in response to acute hypoxia are higher, but the percentage of increase remains equal. Chronic hypoxia produced profound changes in CB weight, and CB CA metabolism, as well as in basal release of DA and adenosine in the CB, these alterations being important to the observed acclimatisation to chronic hypoxia (Table 8). The increased release of adenosine in response to acute hypoxia suggests a role for this nucleotide in the responses to acute hypoxic challenges during chronic hypoxic exposure (Table 8).

Ventilatory acclimatisation to hypoxia (VAH) is defined as the time-dependent increase in ventilatory responses to acute hypoxic challenges lasting from several hours to months (Bisgard, 1994, 1995, 2000; Powell et al., 2000a; Powell, 2007). After 8 and 15 days of hypoxic exposure rats were acclimatised, the acclimatisation being more pronounced after 15 days, as the increase in the HVR is higher than in rats submitted to 8 days of exposure. These results are in agreement with the described VAH in the rat in which breathing increases after some hours or days of hypoxic exposure persisting at least 14 days (Olson and Dempsey, 1978). These acclimatisation phenomena were also observed in the responses of CSN to acute hypoxia in chronic hypoxic rats, in which chronic hypoxia exposure increase basal frequency of discharge and the frequency of action potentials in acute hypoxic challenges but does not change the percentage of increase in CSN discharges, these responses being in accordance with the previous findings by Chen et al. (2002b) and He et al. (2005; 2006) in the rat.

Neurochemical and physiological parameters		**Normoxia + Chronic caffeine**	**Chronic hypoxia (15 days)**	**Chronic hypoxia + Chronic caffeine**
CBs weight		=	↑	=
CA content	NE	=	↑	↑
	DA	=	↑↑↑	↑↑↑
	DA/NE ratio	=	=	↑↑↑
CA synthesis	NE	=	=	↑↑
	DA	=	↑↑↑	↑↑↑
CA turnover time	NE	=	↑	=
	DA	=	=	=
NTs basal release	DA	=	↑↑	↑↑
	ATP	=↑	=	=
	adenosine	=	↓↓	↓↓
NT release with 7% O_2 hypoxia	DA	↑	↓	↑
	ATP	=	↓↓	↑
	adenosine	=	↑↑	↑
NT release with 2% O_2 hypoxia	DA	=	=	=
	ATP		↓↓	↑
	Adenosine	=	↑↑	↑↑
TH expression		=	↑↑	↑↑
CSN chemosensory activity	basal	=	↑↑	↑↑
	5%O_2	= ↑	=	↓↓
	N_2	= ↑	=	↓↓
	20%CO_2	=	↓	↓↓↓
Minute Ventilation	basal	=	↑	↑
	hypoxia	↑↑	↑↑	↑↑
	5% CO_2	=	=	↑↑

Table 8 Effects on neurochemical and physiological parameters that include CB neurotransmitters, ATP, DA and adenosine, dynamics (content, synthesis, basal and evoked release), CSN activity and ventilation in rats submitted to chronic caffeine intake (1g/l) for 15 days, in rats exposed a hypoxic atmosphere of 12% O_2 for 15 days and in rats exposed to both situations. Parameters that are altered when compared with rats exposed to chronic hypoxia are marked in red. ↑, ↑↑, ↑↑↑, ↓ and ↓↓ - correspond to significant increases or decreases in comparison with normoxic rats, ↑, ↑↑, ↑↑↑, ↓↓, and ↓↓↓ - significant increases or decreases in comparison with chronically hypoxic rats.

7.4.1.1. Effect of chronic hypoxia on morphology and neurotransmitter dynamics in the carotid body

As previously described in the General Introduction of this work, chronic hypoxic exposure induces several morphological and neurochemical modifications in the CB, like a hypertrophy and hyperplasia of the CB and an increased CB CA content and turnover time and an increased DA basal release. In the current work we have confirmed these results and we have also observed that chronic hypoxia did not modify ATP basal release, and that it decreases adenosine basal release, these phenomena being accompanied by a maintained and non-significant small decrease in DA release in response to intense and mild hypoxia respectively. Chronic hypoxia also decrease ATP release in response to mild and intense acute hypoxia and produce an increase in the release of adenosine in both acute hypoxic conditions. Increased DA metabolism, as well as TH expression and DA basal release, would increase extracellular levels of DA thereby providing a decrease in D_2-mRNA and/or desensitisation or down-regulation of D_2 receptors, explaining the stimulation of CB chemoreceptors and the increased O_2 sensitivity in chronic hypoxia. However, the maintained DA release in response to intense hypoxia is not compatible with this hypothesis, as well as the finding that mRNA levels of D_2 receptors increase after 7 days of chronic hypoxia (Huey and Powell, 2000). These findings are in accordance with the DA-mediated inhibition of ventilatory response induced by chronic hypoxia observed in rats (Huey et al., 2000) and suggest that the alterations in DA metabolism and release are not sufficient to explain VAH in rats.

The absence of effects of chronic hypoxia in the basal release of ATP and the fact that in chronic hypoxic conditions acute hypoxic tests did not induce the release of ATP from rat CB, suggest that this neurotransmitter is probably not involved in the increased O_2 sensitivity observed in chronic hypoxia, contrary to what has previously been suggested by.He et al. (2006).

Given these differences in the release of DA and ATP in response to acute hypoxia in the CB of chronic hypoxic rats and the knowledge that the release of DA is totally vesicular and ATP is also almost totally vesicular, these

results could suggest that DA turnover time will be higher than ATP turnover time.

The increased release of adenosine in acute hypoxia of mild and intense intensities in chronic hypoxic rats (Table 8) is in agreement with the increased frequency of CSN actions potentials observed in chronic hypoxia and thereby with the increased ventilatory hypoxic response observed in chronic hypoxia. It is important to note that the pattern of increase in adenosine release, that is higher in response to moderate and mild hypoxia than with intense hypoxias (Conde and Monteiro, 2006), in normoxic rats was switched in chronic hypoxia. Thus, in chronic hypoxia, adenosine release from chemoreceptor cells is proportional to stimulus intensity. These findings are consistent with increased adenosine in PC12 cells in chronic hypoxia (Kobayashi et al., 2000b) and suggest a role for adenosine in the enhanced O_2 sensitivity observed in chronic hypoxia. Knowing that adenosine is a product of ATP catabolism, and that in chronic hypoxia the levels of adenosine released in response to acute hypoxia are elevated and the levels of ATP are diminished, we can postulate that this can be due to an increased activity of ecto and endo 5'-nucleotidases with a preferential utilisation of adenosine in these situations. In fact, in PC12 cells, it is known that cytoplasmatic and ecto-5'-nucleotidase activity are increased during chronic hypoxia (Kobayashi et al., 2000).

7.4.1.2. Effect of chronic hypoxia on the carotid body output

Chronic hypoxia, as previously described by other authors (Banard et al., 1987; Nielsen et al., 1988; Vizek et al., 1987), increases CSN basal chemosensory activity. Chronic hypoxia also increases CSN discharges in response to hypoxia, although when the values are corrected to basal activity, the increase is unaltered when compared with normoxic rats. The increase is consistent with an increase in adenosine levels released by acute hypoxic challenges in chronic hypoxia.

Olson and Dempsey (1978) described that chronic hypoxia elicited hyperventilation in the rat over 14 days. We have observed an increase in hypoxic ventilatory response both in rats exposed to 1 week or 2 weeks of chronic hypoxia, and an absence of effect of hypercapnia in eliciting VAH. The

last data are consistent with the earlier findings of Bisgard et al. (1986b) where, using a preparation in which CB was isolated from systemic circulation, they showed that hypercapnia did not induce ventilatory acclimatisation in goats.

7.4.2. Effects of chronic caffeine ingestion on rat CB function

7.4.2.1. In control animals

In this study we have also demonstrated that chronic caffeine intake for 15 days did not alter the CB basal function significantly. In contrast, chronic caffeine intake facilitated the response to hypoxia as it increased minute ventilation and showed a tendency to increase the CSN discharges and DA release, while maintaining adenosine and ATP release induced by acute hypoxia (Table 8).

The absence of effects of chronic caffeine intake in the CB basal conditions in normoxic rats are in agreement with the existence of the minor negative consequences of chronic caffeine intake previously described (see Fredholm et al., 1999). The treatment with chronic caffeine has maintained DA and adenosine dynamics in basal and hypoxic conditions, maintaining also the release of ATP in response to hypoxia, increasing the basal levels of this neurotransmitter's release non-significantly. This increase in CB basal ATP release suggests that adenosine modulates the release of ATP, as happens in the rat medial habenula nucleus (Robertson and Edwards, 1998) and in the vas deferens (Driessen et al., 1994). Therefore, a more extensive study must be performed in order to confirm this hypothesis and to characterise the receptors responsible for this modulation.

The existence of a facilitation in the response to acute hypoxia that is not significant in CSN chemosensory activity but is significant in minute ventilation means that chronic caffeine has an excitatory effect on respiration due to a combined excitatory effect on peripheral and central chemoreceptors, in which central chemoreceptors play a major participatory role. The excitatory effect of caffeine, and thereby inhibitory action of adenosine, in response to hypoxia on central chemoreceptors has been previously described in infants by several authors (Aranda and Turmen, 1979; Bairam et al., 1997; Herlenius and

Langercrantz, 1999). However, it has been suggested that this effect is depressed in adults due to a dominance of peripheral hypoxic response when CB expression is completely mature (Gauda et al., 2004).

The observed inhibitory effects of adenosine mean that the response of peripheral chemoreceptors to hypoxia in rats that ingested chronic caffeine is not preponderant to the central chemoreceptor response. These effects of chronic caffeine in response to hypoxia contrast with the findings that in un-anaesthetised monkeys acute caffeine significantly attenuated CB-mediated hyperventilation while animals were breathing 10% O_2 (Howell and Landrum, 1995), but are in agreement with the described opposite effects of acute and chronic treatments with caffeine (Jacobson et al., 1996) observed, for example, in brain ischaemia (Dux et al., 1990; Rudolphi et al., 1989).

7.4.2.2. In chronic hypoxic rats

In order to assess the role of adenosine in VAH to chronic hypoxia we permanently blocked adenosine receptors with chronic caffeine ingestion. When rats were submitted to chronic caffeine intake during chronic hypoxia they developed even higher increases in CA metabolism. The application of both treatments together did not alter the basal CSN chemosensory activity but diminished the CSN discharges in response to hypoxia. The observed excitatory effect of adenosine (inhibitory effect of chronic caffeine) in CB output is compensated at central chemoreceptors as the minute ventilation in basal conditions and in response to hypoxia remains unaltered in rats exposed to chronic hypoxia (Table 8).

The first fact that we want to discuss is the partial reversion of the hyperplasia of CB observed in chronic hypoxic animals. It is known that adenosine is involved in neovascularisation and angiogenesis via A_{2A} and A_{2B} receptors in endothelial cells in pigs, rats and humans (see e.g. Dubey et al., 2002; Montesinos et al., 2004) and it is known that chronic hypoxia induces the formation of new vessels in the CB (Laidler and Kay, 1978; McGregor et al., 1984; Pequinot et al., 1984; Heath et al., 1985) via an increased expression of VEGF. The fact that caffeine, which is a non-selective adenosine receptor

antagonist, abolishes almost completely all the CB hyperplasia means that adenosine could be mediating the neovascularisation and therefore the hyperplasia of the CB in chronic hypoxia.

The second point to discuss is the higher increase in CA metabolism in the CB of chronic hypoxic rats with chronic caffeine ingestion (Table 8). These significant increases in DA content, synthesis, DA/NE ratio and DA endogenous release in response to mild hypoxia can be explained by the permanent blockage of A_{2B} adenosine receptors present in chemoreceptor cells that control the release of dopamine from these cells (Chapter 3, Section 5). If A_{2B} receptors were blocked, D_2 receptors could exhibit their maximal inhibitory pattern, decreasing the DA released from chemoreceptor cells. Thus, through the negative feedback mechanism, the response of the cell would be an increase in DA synthesis in order to obtain a higher DA content to release more DA, as is observed in our results (Table 8). The increase in DA endogenous release that is observed in response to a mild hypoxia but not in response to an intense hypoxia can be explained by the interaction between A_{2B}-D_2 receptors being functional at moderate and mild hypoxias (Chapters 3 and 4, Section 5 and 6) i.e. in hypoxias that are compatible with life, but not being responsible for controlling the release of dopamine in response to an intense stimulus. These results lead us to the conclusion that the interaction between A_{2B} and D_2 receptors is present, being functional in chronic hypoxic rats and contributing to the control of DA release in this situation. The fact that chronic caffeine increases DA metabolism and release in chronic hypoxia, through the permanent inhibition of A_{2B} - D_2 receptor interaction, can be extremely important, since, pathologically, a correlation between caffeine intake and the increased CA levels is described in sleep apnoea (Bardwell et al., 2000).

Chronic treatment with caffeine did not alter ATP basal release from rat CB but reversed the reduction of ATP levels released from CB in response to acute hypoxic stimulus during chronic hypoxia. These results suggest that, during chronic hypoxia, adenosine inhibits the release of ATP from rat CB probably via A_{2A} receptors, as described in the rat medial habenula nucleus (Robertson and Edwards, 1998). A more detailed study with specific pharmacological tools must be performed in order to study how adenosine

controls the release of ATP during chronic hypoxia and to assess the role of ATP itself on the hypoxic ventilatory response in chronic hypoxia.

Chronic caffeine intake in chronic hypoxia did not alter basal release of adenosine or the release evoked by mild or intense hypoxia in comparison with rats exposed to chronic hypoxia, suggesting that adenosine is not responsible for its own release i.e. that the release of adenosine from chemoreceptor cells does not seem to be totally controlled by a negative feed-back mechanism through adenosine receptors. These results are in agreement with the previous findings that acetylcholine via nicotinic receptors with α4 subunits modulate, in part, the release of adenosine from CB (Chapter 2, Section 4).

Basal CSN chemosensory activity observed in chronically hypoxic rats was unaltered with the chronic caffeine ingestion. These data conform with those previously demonstrated, that in normoxic rats acute application of caffeine did not alter the basal chemosensory activity of CSN (Chapter 3, Section 5). As occurs with acute caffeine application in normoxic rats, chronic caffeine significantly attenuatedthe CSN responses to acute hypoxic and hypercapnic challenges by approximately 60% and 75% (Table 8), respectively, in chronic hypoxic rats. Therefore we can establish a correlation between the acute application of caffeine in normoxic rats and the chronic application of caffeine in chronic hypoxic rats, and say that possible the adenosine effects observed (i.e. caffeine inhibition of CSN chemosensory activity) in chronic hypoxic rats are due to an action on A_{2A} and A_{2B} adenosine receptors in the CB. These data demonstrate that adenosine is essential for the CB chemoreception in chronic hypoxia, being one of the neurotransmitters involved in the increased O_2 sensitivity and contributing to the VAH. These roles are in agreement with the suggestion that adenosine released in acute hypoxia could stimulate A_2 receptors in the CB to increase ventilation (Walsh and Marshall, 2006) in chronic hypoxic rats.

Despite the reduction in CSN chemosensory activity in response to acute hypoxia and hypercapnia, similar increases in hypoxic ventilatory responses were observed in chronic hypoxic rats that ingested caffeine chronically (table Table 8). However, acute hypercapnic challenge was capable of inducing VAH

in these rats. These results suggest that VAH in chronic caffeine rats is compensated by central chemoreceptors, this suggestion being compatible with the VAH elicited by the acute application of hypercapnia. An enhanced responsiveness of CSN respiratory centres have been described as an increase in CSN gain in the hypoxic ventilatory response (Powell et al., 2000b), as doxapram (chemoreceptor stimulant) increases ventilation in subjects acclimatised for 2-3 weeks, this effect being due to greater ventilatory output of CNS, since doxapram effect on CB is unchanged by chronic hypoxia (Forster et al., 1974). An increased CNS gain of the hypoxic ventilatory response was also observed in phrenic nerve activity measurements in rats in which chronic hypoxia increased the CNS gain (Dwinell and Powell, 1999). Therefore, chronic caffeine can interfere with the chronic hypoxic interactions between central and arterial chemoreflexes, allowing central chemoreceptors to exert a more pronounced effect and compensate the inhibition of arterial chemoreceptors in order to produce the same increased hypoxic ventilatory response.

In conclusion, adenosine is essential for CB chemoreception in chronic hypoxia, being one of the neurotransmitters involved in the increased O_2 sensitivity and contributing to the VAH. Adenosine is involved in the neovascularisation and remodelling that is responsible for CB hyperplasia observed during chronic hypoxia. Adenosine release in response to acute hypoxia is increased in chronic hypoxia and contributes to CSN chemosensory activity elicited by acute hypoxia and thereby to the increased hypoxic ventilatory response. Our data establish a role for adenosine as one of the neurotransmitters responsible for VAH in chronic hypoxia.

8. GENERAL DISCUSSION

Adenosine both in acute and chronic hypoxic conditions has, by itself, an excitatory role in CB chemosensory activity and contributes to the chemoexcitatory effect of other neurotransmitters like ACh and ATP. It acts directly on adenosine A_{2A} receptors present postsynaptically in CSN and it controls the release of dopamine presynaptically via A_{2B} receptors in chemoreceptor cells. We also conclude that A_{2B} -D_2 adenosine/dopamine interactions at the CB could explain the increase in CA metabolism caused by chronic ingestion of caffeine during chronic hypoxia.

Knowing that the carotid body contains several neurotransmitters, namely ACh (Fitzgerald and Shirahata, 1996; Fitzgerald, 2000), NE (Mir et al., 1982), DA (Gonzalez et al., 1994), SP (McQueen, 1980; Hanson et al., 1986), ATP (Buttigieg and Nurse, 2004; Conde and Monteiro, 2006b) and adenosine (Monteiro and Ribeiro, 1987, 1989; Runold et al., 1990) and since, at the moment, there does not exist a consensus on the precise role of each substance as mediator between chemoreceptor cells and the sensory endings, it is important to aim to clarify the role of adenosine in response to hypoxia, both in control and in chronic hypoxic animals. In this work we have tested the hypothesis that adenosine is a “synaptic transmitter” at the chemoreceptor cell/sensory fibre synaptic complex of the carotid body. We have collected evidence that fulfill the criteria (McLennan, 1963) for adenosine as a primary neurotransmitter.

The first of these criteria reads: *A substance must occur in sufficient quantity in structures from which it will be released.* Adenosine is present in all cells. It is an ATP precursor and an ATP catabolite and therefore it is not necessary to demonstrate its presence in the chemoreceptor cells. However, in Chapter 1, Section 3 we have reported that adenosine is released in basal conditions by CB, this release being increased in conditions of presynaptic activation (Figure 17), like hypoxia. We have also described a reduction in adenosine CB content during hypoxia (Figure 17). Since adenosine is present in all cells, it is possible that hypoxia can also release this mediator from other CB cell types aside from chemoreceptor cells. However, as we have shown (Figure

19), moderate hypoxia is not a strong enough stimulus to evoke adenosine release from other cell types. It is reasonable to assume that it is the pool of adenosine present in chemoreceptor cells that is reduced after hypoxia.

The second criterion requires: *The structures must possess an enzyme system for synthesis of the transmitter*. Adenosine can be synthesized extracellularly or intracellularly by ATP catabolism through nucleotidases (Latini and Pedata, 2001; Zimmermann et al., 1998) or by the hydrolysis of S-adenosylhomocysteine (Figure 9). Beyond ATP catabolism, another source of extracellular adenosine is cAMP that can be released by secretory cells and converted by extracellular phosphodiesterases in AMP and then by a 5'-ectonucleotidase into adenosine (for a review see Fredholm et al., 2001). It is known that ATP is present in the CB in the rat (Buttigieg and Nurse, 2004; Conde and Monteiro, 2006), in the cat (Acker and Starlinger, 1984; Obeso et al., 1986) and in the rabbit (Verna, 1990) being released in response to acute hypoxia (Figure 54, Buttigieg and Nurse, 2004; Conde and Monteiro, 2006). The presence and CB content of cAMP has also been described in several species, such as the rat (Batuca et al., 2003; Chen et al., 1997; Monteiro et al., 1996) and rabbit (Perez-Garcia et al., 1990; 1991) and it is known that CB cAMP content increases in response to CB physiological stimulus, hypoxia (Perez-Garcia et al., 1990; 1991; Chen et al., 1997; Batuca et al., 2003) that can lead to an increased production of adenosine. In this work we have shown that the blockade of ATP extracellular catabolism reduced the levels of adenosine present in incubating milieu both in normoxia and hypoxia (Figure 21). The reuptake mechanisms, defined in the case of adenosine as a bi-directional equilibrative nucleoside transport system, are present in chemoreceptor cells, as the concentrations of adenosine released from CB were modified in the presence of ENT inhibitors (Figure 21). Therefore, it can be assumed that chemoreceptor cells possess adenosine synthesising enzymes.

A third criterion requires: *There must be an enzymatic system for inactivation of the transmitter.* Adenosine has a short half-life (<10 s) (Moser et al., 1989) and is converted by adenosine deaminase (EC 3.5.4.4) to inosine. Adenosine deaminase is present in the CB as the concentrations of adenosine released from CB are higher in the presence of the adenosine deaminase inhibitor, EHNA (Conde and Monteiro, 2002). The reuptake mechanisms, as

described above in criterion 2, are also present in the CB, as the concentrations of adenosine released from CB were modified in the presence of ENT inhibitors (Figures 20 and 21). Enhanced postsynaptic effects when the inactivation enzymes (ADA) or reuptake mechanisms (ENT) are blocked have been previously described by Monteiro and Ribeiro (1989) *in vivo* in the rat, where EHNA and dipyridamole mimicked the excitatory effect of adenosine on respiration, an effect attributed to CB chemoreceptors since it is abolished after CSN section.

The fourth criterion requires that: *Small amounts of suspected neurotransmitter must mimic the action of normal stimuli*. By recording the primary reflex response, ventilation, or neural activity from chemoreceptor fibres in the CSN, it has previously been shown that exogenously applied adenosine and inhibitors of adenosine metabolism mimic the responses generated by hypoxia (McQueen and Ribeiro, 1983; Monteiro and Ribeiro, 1987; 1989; Runold et al., 1990).

A fifth criterion requires: *During activity the transmitter should be detectable in extracellular fluid around the synapses.* This work (Chapter 1, Figure 17) reports the first evidence that adenosine is released from CB. Adenosine is released during normoxic conditions and the amount released increases significantly under acute hypoxic conditions both in control animals and in chronic hypoxic animals (Figures 17 and 55; Conde and Monteiro, 2006b).

A sixth criterion requires: *Postsynaptic actions can be antagonised with agents considered to be specific to the transmitter's receptors.* In this work we have used specific and non-specific antagonists to the several subtypes of adenosine receptors on the neural chemosensory activity of CSN and in all cases we found that these antagonists decreased the CSN chemosensory activity induced by acute hypoxia, and therefore, by adenosine (Figures 36 and 37). The same inhibition of CSN chemosensory activity was also observed in chronic hypoxic animals treated with caffeine (non-selective antagonist of adenosine receptors), in which animals respond less to acute hypoxia and to hypercapnia than control rats (Figure 57).

The last criterion requires: *Actions of the transmitter should be enhanced by drugs that block inactivating enzymes*. Monteiro and Ribeiro (1989) have

observed that the exogenous excitatory effect of adenosine on ventilation is mimicked by EHNA and dipyridamole, inhibitors of adenosine deamination and uptake, respectively.

Fulfilment of these criteria leads us to the conclusion that adenosine is a primary transmitter in the hypoxic CB chemoreception process (Figure 61). Apart from its role in hypoxic chemotransduction, adenosine also seems to be involved in the chemoreception mechanism initiated by substances like nicotine and acetylcholine that apparently mimic the excitatory effects of hypoxia on CSN activity and/or ventilation. It would be important to know if adenosine is also involved in the responses of CB to hypercapnia/acidosis. In this work we have focused only on the functional significance of adenosine in the hypoxic chemosensory process in control and chronic hypoxic animals, in view of the fact that hypoxia is the most important physiological stimulus of CB chemoreceptors and previous findings suggested a role for this substance in hypoxia. Nevertheless, to develop this work it would be important to investigate the functional significance of adenosine in the hypercapnic/acidosis chemoreception processes in order to establish a role for adenosine as a primary neurotransmitter in CB chemoreceptors for all natural stimuli.

Apart from this role as primary neurotransmitter, adenosine in the CB also works as neuromodulator, since it controls the release of CA from chemoreceptor cells via A_{2B} adenosine receptors (Figure 61) in control rats and probably in rats exposed to chronic sustained hypoxia. Therefore, adenosine is involved in the response of chemoreceptor cells to hypoxia both pre- and post-synaptically, controlling the release of other neurotransmitters and also acting directly on its receptors, A_{2A}, to stimulate CSN fibres to produce the central hyperventilatory response. Nevertheless, A_{2A} adenosine receptors present at postsynaptic level were only characterised pharmacologically and it would be interesting perform experiments in order to visualise these receptors using confocal microscopy or electron microscopy in the CSN.

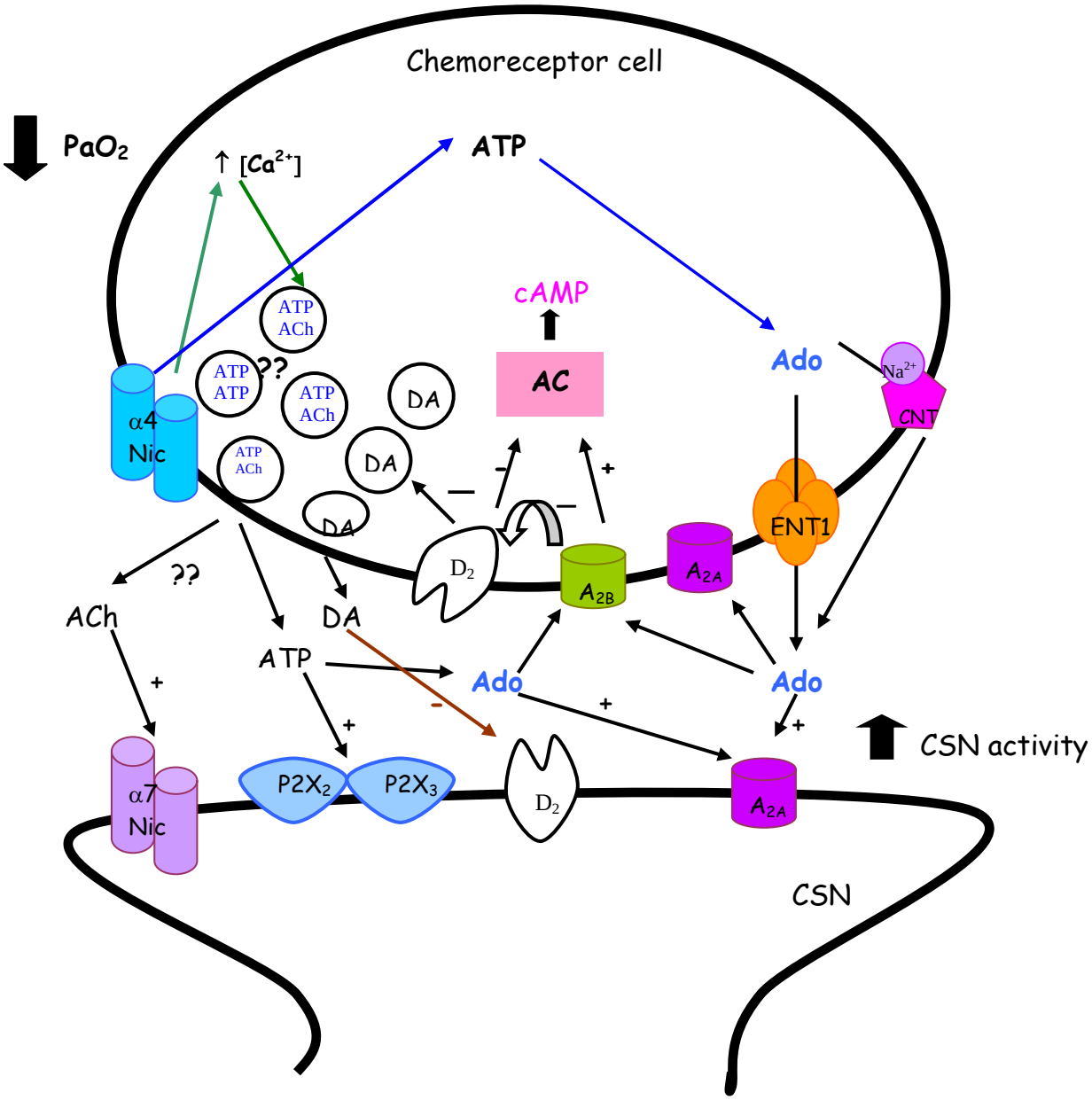


Figure 60 The possible mechanism of action of neurotransmitters involved in the chemosensory response to acute hypoxia in the carotid body. AC – adenylyl cyclase; Ado – adenosine; CSN – carotid sinus nerve; DA – dopamine; ENT1 – Equilibrative nucleoside transport system type I; Nic – nicotinic receptors.

Recently it has become consensual that ATP and ACh are the two neurotransmitters responsible for hypoxic CB chemotransduction, in view of the fact that application of nicotinic and P2 receptor antagonists clearly block the postsynaptic CB hypoxic response, suggesting that ATP and ACh are co-released from chemoreceptor cells (Zhang et al., 2000). In fact, the existence of nicotinic receptors (Shirahata et al., 1998) and P2X2/P2X3 (Nurse and Zhang,

2001; Prasad et al., 2001) at postsynaptic level in the petrosal ganglion suggest that ATP and ACh could be the primary neurotransmitters responsible for the hypoxic chemoresponse. Nevertheless, more recently it has been suggested that ACh in the CB apparently comes from nerve endings instead of chemoreceptor cells, since the mRNA of ChAT was not detected in the chemoreceptor cells, but in the sensory nerve endings, autonomic microganglia and in petrosal neurons (Gauda, 2002; Gauda et al., 2004) and therefore the action of ACh could be on nicotinic receptors present in chemoreceptor cells, such as nicotinic receptors with α_4 subunits (Chapter 2, Section 4). In addition, it has been shown that the combined blockade of nicotinic and purinergic receptors only reduce the CB chemosensory responses to hypoxia by 50% and 60% (Reyes et al., 2007a; 2007b). Zapata (2007) suggesting that ATP is indeed an excitatory agent for carotid body chemoreceptors, and that that the joint participation of ACh and ATP contributes to, but does not entirely explain, the transfer of information between chemoreceptor cells and sensory nerve endings. Therefore, it is reasonable to postulate that ACh will act as neuromodulator of adenosine and ATP release, adenosine and ATP being the primary neurotransmitters responsible for CB acute hypoxic responses. However no experiments have been yet performed in the presence of both adenosine and ATP (non-hydrolysable) antagonists in acute hypoxic conditions in order to see if a complete blockade of CSN chemoresponses is achieved.

Up to this point in the General Discussion of this work we have focused on the role of adenosine CB chemosensory response to acute hypoxia because the greatest part of the study was performed to investigate the functional significance of adenosine in this situation. However, in the last chapter we have started to look at the role of adenosine CB chemosensory activity in chronic hypoxic rats.

Basic/experimental approaches to chronic hypoxia are based on the period of time that an individual is submitted to low PaO_2. However, this may not translate directly into clinical situations where chronic hypoxia is interpreted in terms of reversibility of the low PO_2. In our studies we have considered chronic hypoxia as a period of 8 or 15 days and we should mention that in

clinical practice this period of time corresponds, for example, to some pneumonia situations or to the acute respiratory distress syndrome, that are considered acute respiratory failures (Hudson and Steinberg, 1998).

Furthermore we should point out that we have used these periods of time because after some hours of low PO_2 gene expression has been already apparent, leading to profound morphological as well as biochemical changes in the CB, that are consistent with an increased sensitivity of CB chemoreceptors to acute hypoxia (Barnard et al., 1987) and with some pathological conditions, like COPD, asthma or pulmonary fibrosis.

At the outset of the discussion of our data on chronic hypoxia we should state that we have attributed all the effects of chronic caffeine ingestion during chronic hypoxia to an inhibition of adenosine receptors, since the concentrations of caffeine ingested are compatible with this inhibition and not with other cell targets (Section 1.7). In accordance with our statements, the implication is that all the effects of caffeine observed are due to an inhibition of adenosine receptors and therefore, in the rest of the discussion we will not make a specific distinction between the effects of caffeine or adenosine.

Knowing that chronic hypoxia produces changes in the CB at several levels, neurochemical, histological, etc., it is possible that these neurotransmitters do not have the same importance in the hypoxic chemotransduction process in chronic hypoxic animals. Our results led us to the conclusion that adenosine is involved in the CB sensitisation observed during chronic hypoxia, since caffeine, an adenosine receptor antagonist, produced neurochemical alterations in CB that led to decreased CSN activity in response to hypoxia and hypercapnia. Therefore, we can suggest that adenosine is also a key neurotransmitter contributing to the hypoxic chemoreception process in the CB of chronic hypoxic animals. The results obtained in this thesis are in apparent contradiction with the results obtained by He et al. (2006). They suggest that ATP is one of the neurotransmitters that contribute to ventilatory acclimatisation to hypoxia observed during chronic hypoxia, since we have observed that acute hypoxic stimuli of moderate and high intensity did not induce ATP release in chronic hypoxic animals. The fact that chronic hypoxia abolished acute hypoxic ATP release means that ATP cannot be one of the

neurotransmitters involved in the increased hypoxic sensing observed during chronic hypoxia.

In chronic hypoxic animals adenosine is responsible for nearly 60% of the activity elicited by hypoxia, this value being higher than that observed for the contribution of this mediator to the hypoxic chemotransduction process in control animals (≈ 50%) (Chapter 3, Section 5). Nevertheless, since caffeine cannot completely abolish the hypoxic CSN chemosensory activity, other excitatory neurotransmitter(s) must be involved in the hypoxic chemosensory process during chronic hypoxia. The results obtained by He et al. (2005) have excluded ACh as an important neurotransmitter in chronic hypoxia, since nicotinic and muscarinic antagonists failed to decrease hypoxic CSN chemosensory activity and the results presented here exclude ATP as the primary neurotransmitter involved in acclimatisation. Therefore, experiments must be performed in order to find the neurotransmitter(s) that, with adenosine, is responsible for the hypoxic chemosensory process during VAH.

We cannot conclude the discussion of this work without focus on its limitations. The major limitation was the lack of studies on expression of the different subtypes of adenosine receptors, nucleoside transporters and nucleotidases during chronic hypoxia and chronic caffeine consumption. This point is extremely important since it is of common knowledge that chronic treatments alter, down- or up-regulating, the expression of several proteins. In our point of view, would be extremely important to perform studies on the expression of A_{2A} and A_{2B} receptors, ENT1 and ENT2 equilibrative nucleoside transporters and expression of 5'-ectonucelotidase as well as cytoplasmatic nucleotidases, in order to look more closely at adenosine metabolism and transport during chronic hypoxia and in presence of chronic caffeine, in order to consolidate the role of adenosine in the acute hypoxic response. This issue will be addressed in the near future.

In order to establish a role for adenosine in the carotid body and not only in chemoreception in response to hypoxia, we must investigate the role of adenosine in CB responses to hypercapnia and acidosis as well. Therefore, the same neurochemical approaches, like the study of adenosine metabolism,

transport and release must be taken in order to see if adenosine is involved in the response to acute hypercapnic and acidosis stimuli.

Knowing that adenosine is one of the mediators involved in the hypoxic ventilatory response observed during chronic hypoxia and since the involvement of ACh and ATP in the VAH have been excluded, we have to explore the possibility of SP or 5-HT as neurotransmitters involved in the hypoxic ventilatory response in chronic hypoxia.

Finally we should mention that this work consolidates the role of adenosine as a primary neurotransmitter in hypoxic sensitivity of CB and opens up new possibilities for the role of adenosine in chemoreception in several CB-associated pathologies, leading us to several future projects. It is known that patients with OSA are high consumers of caffeine and that in an initial phase of the disease try to pass up the symptoms with an increased coffee ingestion. Several times, caffeine consumption has been correlated with elevated blood pressure (Jeong and Dimsdale, 1990; Green et al., 1996; Rakic et al., 1999) and there is a suggestion that caffeine augments blood pressure and total peripheral resistance in response to stressors (Sung et al., 1994; Pincomb et al., 1996). Nevertheless, more recent studies suggest that caffeine does not seem to be a factor that contributes to the positive correlation between OSA and hypertension (Bardwell et al., 2000; Robinson et al., 2003) although it remains unclear if coffee contributes to the hypertension observed in this disease. An augmented carotid body sensory response to acute hypoxia has been observed in adults suffering from OSA (Peng and Prabhakar, 2004; Rey et al., 2004), like the response observed here in chronic sustained hypoxia. We also know that increased CB activity leads to an increased sympathetic activation that produces hypertension (Timmers et al., 2004). If the mechanisms underlying the increased hypoxic sensitivity during chronic intermittent hypoxia were similar to those observed during chronic sustained hypoxia, adenosine would be one of the neurotransmitters responsible for this increased sensitivity, explaining the increased plasma levels of adenosine observed in OSA patients (Saito et al., 2002), and the ingestion of caffeine would not contribute to the hypertension observed in patients with OSA. In order to test these hypotheses the same experimental design that we have applied to study the role of

adenosine in chronic sustained hypoxia must be performed in animals submitted to chronic intermittent hypoxia.

9. CONCLUSIONS

1. CB chemoreceptor sensitivity to hypoxia could be related to its low threshold for the release of adenosine because:

    a. Moderate acute hypoxia (10% O_2) increased adenosine concentrations released from the CB by 44% but was not a strong enough stimulus to evoke adenosine release from SCG and arterial tissue;

    b. In the CB approximately 40% of extracellular adenosine came from extracellular catabolism of ATP, both under normoxic (20% O_2) and moderate hypoxic conditions. Low PO_2 conditions trigger adenosine release through activation of the NBTI-sensitive equilibrative nucleotide transport system.

2. ACh modulates the release of adenosine/ATP from CB in moderate hypoxia suggesting that the excitatory role of ACh in chemosensory activity includes indirect activation of purinoreceptors by adenosine and ATP, which strongly supports the hypothesis that ATP/adenosine are important mediators in chemotransduction, since:

    a. The activation of ACh nicotinic receptors at the CB in normoxia stimulates the release of adenosine (max 36%) that apparently comes mainly from extracellular degradation of ATP. The increase in the amount of adenosine in the CB induced by hypoxia was partially antagonised by ACh nicotinic receptor antagonists.

    b. The pharmacological characterisation of the ACh nicotinic receptors involved in the stimulation of adenosine release revealed that ACh nicotinic receptors with α_4 and $ß_2$ receptor subunits are involved.

3. Adenosine modulates the release of CA from rat CB chemoreceptor cells via A_{2B} receptors, based on the following data:

    a. Caffeine, a non-selective antagonist of adenosine receptors, inhibits the CA normoxic release and that evoked by stimuli of low intensity, being ineffective on the release elicited by stronger stimuli.

    b. The effectiveness of DPCPX, MRS 1754 in mimicking caffeine effects and the absence of effect of SCH 58621 on the induced release indicated that caffeine effects were mediated by A_{2B} receptors present

presynaptically, since they co-localise with tyrosine hydroxylase in chemoreceptor cells in rat CB.

4. Acute caffeine inhibited the CSN chemosensory activity elicited by hypoxia by 52%, an effect mediated by postsynaptic A_{2A} and presynaptic A_{2B} adenosine receptors, indicating that chemosensory activity elicited by hypoxia is controlled by adenosine.

5. The theory of an antagonistic interaction between A_{2B} and D_2 receptors, described in the CB for the first time, was supported by the findings that:

a. The dopamine D_2 antagonist, sulpiride, increased the release of CA from chemoreceptor cells and attenuated the inhibitory effect of caffeine on CA release;

b. The dopamine D_2 antagonists, domperidone and haloperidol, increased basal and evoked release of CA from chemoreceptor cells confirming the presence of D_2 autoreceptors in the rat CB that control the release of CA through a negative feed-back mechanism;

c. Propylnorapomorphine, a D_2 agonist, inhibited basal and hypoxic release of CA, this effect being attenuated by NECA, a non-selective adenosine agonist.

The interaction between A_{2B} and D_2 receptors can also occur at second messenger level, as it was observed that NECA potentiates the effect of haloperidol on the release of CA from chemoreceptor cells both in basal conditions and in response to moderate hypoxia.

6. Using a combination of neurochemical and physiological techniques we have demonstrated that chronic caffeine treatment in normoxic rats does not significantly alter the basal function of CB, assessed as the dynamics of their neurotransmitters, dopamine, ATP and adenosine, and the CSN chemosensory activity. In contrast, chronic caffeine intake facilitated the response to hypoxia as it increased the ventilatory response and showed a tendency for CSN chemosensory activity to increase and increase DA and ATP release.

7. After a period of 15 days of chronic hypoxia, acclimatisation occurs as ventilatory hypoxic response was increased and CSN basal discharges and in response to hypoxia were increased. The alterations in DA metabolism, as well as in the CB basal release of DA, ATP and adenosine, could contribute to the observed acclimatisation to chronic hypoxia. The increased release of

adenosine in response to acute hypoxia suggests a role for this mediator in maintaining and increasing the ventilatory responses to acute hypoxic challenges during chronic hypoxia. Acute hypoxia-induced release of ATP was diminished in CH. Nevertheless, chronic caffeine treatment brings ATP release in acute hypoxia in CH to levels similar to the control, suggesting that adenosine can modulate the release of ATP during chronic hypoxia.

8. CA metabolism, assessed as expression of TH, and content, synthesis and release of CA, was increased in chronic hypoxic rats also submitted to 15 days of chronic caffeine treatment.

9. Chronic caffeine treatment did not alter the basal CSN chemosensory activity in chronic hypoxic rats but the responses to mild and intense hypoxia and hypercapnia were diminished. This inhibitory effect of chronic caffeine on CB output is compensated for by central mechanisms, as the minute ventilation parameter in basal conditions and in response to acute hypoxic challenges remains unaltered in rats exposed to chronic hypoxia for 15 days.

In summary:

1 – In both acute and chronically hypoxic conditions, adenosine has an excitatory role in the CB chemosensory activity, acting directly on adenosine A_{2A} receptors present postsynaptically in CSN, as well as controlling the release of dopamine presynaptically via A_{2B} receptors in chemoreceptor cells.

2 – A_{2B} -D_2 adenosine / dopamine interactions at the CB could explain the increase in CA metabolism caused by chronic ingestion of caffeine during chronic hypoxia.

3 – In terms of ventilatory response to acute hypoxia during acclimatisation to hypoxia, the inhibitory action of caffeine mediated by peripheral chemoreceptors was compensated for by its excitatory effect at SNC.

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